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(21) International Application Number: PCT/US96/15934 (22) International Filing Date: 2 October 1996 (02.10.96) (30) Priority Data: 08/539,097 4 October 1995 (04.10.95) US (71) Applicant: EPOCH PHARMACEUTICALS, INC. [US/US]; 1725 220th Street, S.E., No. 104, Bothell, WA 98021 (US). (72) Inventors: KUTYAVIN, Igor, V.; 16520 North Road #B101, Bothell, WA 98012 (US). WOO, Jinsuk; 3730 204th Street S.W. #C-201, Lynnwood, WA 98036 (US). LUKHTANOV, Eugeny, A.; Apartment #E-206, 16520 North Road, Bothell, WA 98012 (US). MEYER, Rich, B., Jr.; 15533 61st Avenue N.E., Bothell, WA 98011 (US). GAMPER, Howard, B.; 14048 212th Drive N.E., Woodinville, WA 98078 (US). (74) Agents: KLEIN, Howard, J. et al.; Klein & Szekeres, L.L.P., Suite 700, 4199 Campus Drive, Irvine, CA 92612 (US).		(81) Designated States: AL, AM, AU, AZ, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: SELECTIVE BINDING COMPLEMENTARY OLIGONUCLEOTIDES		
(57) Abstract In a matched pair of oligonucleotides (ODNs) each member of the pair is complementary or substantially complementary in the Watson Crick sense to a target sequence of duplex nucleic acid where the two strands of the target sequence are themselves complementary to one another. The ODNs include modified bases of such nature that the modified base forms a stable hydrogen bonded base pair with the natural partner base, but does not form a stable hydrogen bonded base pair with its modified partner. This is accomplished when in a hybridized structure the modified base is capable of forming two or more hydrogen bonds with its natural complementary base, but only one hydrogen bond with its modified partner. Due to the lack of stable hydrogen bonding with each other, the matched pair of oligonucleotides have a melting temperature under physiological or substantially physiological conditions of approximately 40 °C or less. However each of the matched ODN pair of the invention forms a substantially stable hybrid with the target sequence in each strand of the duplex nucleic acid. The hybrids of target duplex nucleic acids formed with the ODN pairs of the invention are useful for gene mapping and in diagnostic and therapeutic applications.		

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1 SELECTIVE BINDING COMPLEMENTARY
2 OLIGONUCLEOTIDES

3 BACKGROUND OF THE INVENTION

4
5 1. Field of the Invention

6 The present invention is directed to oligonucleotides which
7 include modified bases such that members of a matched pair of the
8 oligonucleotides are unable to form stable hybrids with one another and
9 yet are able to form stable, sequence specific hybrids with
10 complementary unmodified DNA or RNA strands. The present
11 invention is also directed to the use of such oligonucleotides as an
12 anti-sense and anti-gene agents and probes for specific sequences in
13 single or double stranded DNA or RNA.

14 2. Brief Description of Prior Art

15 It is well known that oligonucleotides (ODNs) do not readily
16 hybridize to complementary sequences in double stranded DNA or in
17 DNA or RNA secondary structure. Nevertheless, it is also known that
18 the ability to sequence specifically access double stranded DNA or
19 single stranded RNA or DNA in secondary structure would have great
20 utility in gene mapping, diagnostics and therapeutic applications.
21 Methods known in the prior art which, although limited in scope,
22 accomplish hybridization of ODNs to duplex nucleic acids include
23 triplex formation (see Troel, S. et al. Science 1991, 254, 1639), the
24 branch capture reaction (Weinstock, P. et al. Nucl. Acids Res. 1990, 18,
25 4207), recombinase mediated synapsis (Roca, A. I.; et al. Rev. Biochem.
26 Mol. Biol. 1990, 25, 415) and cross-linking of the hybridized ODN to at
27 least one strand of the duplex nucleic acid (PCT application WO
28 93/03736, published March 4, 1993).

29 There is however still a significant need, and room for
30 improvement in the art, for oligonucleotides which are able to sequence
31 specifically hybridize to duplex nucleic acids. The present invention
32 provides such oligonucleotides.

SUMMARY OF THE INVENTION

In accordance with the present invention a matched pair of oligonucleotides (ODNs) are provided where each member of the pair is complementary or substantially complementary in the Watson Crick sense to a target duplex sequence. However the ODNs include modified bases of such nature that the modified base forms stable hydrogen bonded base pairs with the natural partner base, but does not form stable hydrogen bonded base pairs with its modified partner. Generally speaking, this is accomplished when in a hybridized structure the modified base is capable of forming two or more hydrogen bonds with its natural complementary base, but only one or no hydrogen bonds with its modified partner. Thus, the matched pair of oligonucleotides in accordance with the present invention do not form substantially stable hydrogen bonded hybrids with one another, as manifested in a melting temperature (under physiological or substantially physiological conditions) of approximately 40°C or less. The ODNs of the invention, however, form substantially stable hybrids with the target sequence in each strand of duplex nucleic acid. Due to the increased (approximately double) number of hydrogen bonds in such hybrids (when compared to hybrids that would be formed between a single ODN and duplex nucleic acid) the hybrids formed with the ODN pairs of the present invention are more stable, and lend themselves for gene mapping, diagnostic and therapeutic use. The ODNs of the present invention are termed Selective Binding Complementary (SBC) ODNs, and may be referred to under that name in this application for patent.

The SBC ODNs of the present invention may optionally be connected to one another with a covalent "tether" of such nature that the tether does not prevent hybridization of each ODN to one strand of the target sequence. The SBC ODNs of the present invention may optionally include modifications in the sugar moiety, in the phosphate backbone, and may have cross-linking groups and/or reporter groups

1 attached.

2 **DETAILED DESCRIPTION OF THE INVENTION**

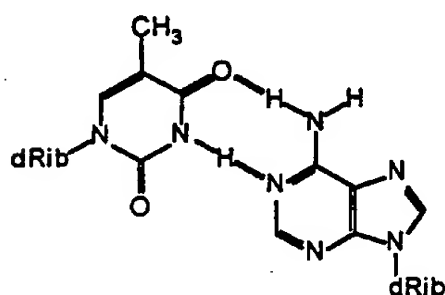
3 As is noted in the Summary of the present application, a key
4 feature of the SBC ODNs of the present invention is that each one of a
5 matched pair of the SBC ODNs is complementary, or substantially
6 complementary, to one target sequence in duplex nucleic acid wherein
7 the target sequences are themselves complementary or substantially
8 complementary to one another, and each one of the matched pair of
9 SBC ODNs forms a stable hydrogen bonded hybrid with one strand of
10 the target sequence. Due to the presence of modified bases in the SBC
11 ODN, although these ODNs are complementary to one another, they
12 are unable to form a stable hydrogen bonded hybrid, as manifested by a
13 melting temperature of approximately 40°C or less. Thus, the SBC
14 ODNs are not hybridized to one another but they readily hybridize,
15 especially in the presence of recombinase enzymes when the target is in
16 long double stranded DNA, with both strands of the target sequence.

17 In accordance with well established convention in the art, the
18 naturally occurring nucleotide components of nucleic acids have the
19 designation A, U, G and C, (RNA) and dA, dT, dG and dC (DNA).
20 As it will become apparent from the following description, the present
21 invention applies to both ribonucleotides and deoxyribonucleotides, and
22 therefore, unless the context otherwise requires, no distinction needs to
23 be made in this description between A and dA, U and dT, etc.

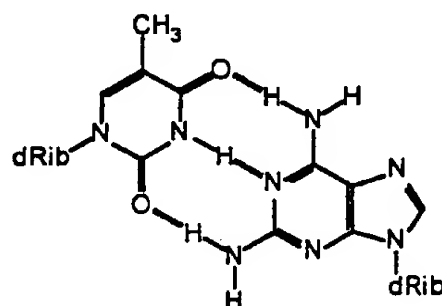
24 Analogs of A which are modified in the base portion to form in
25 an ODN-to-nucleic acid or ODN-to-ODN interaction a stable hydrogen
26 bonded pair with T, (or U in the case of RNA) but not with T' are
27 designated A'. Analogs of T which are modified in the base portion to
28 form in an ODN-to-nucleic acid or ODN-to-ODN interaction a stable
29 hydrogen bonded pair with A, but not with A' are designated T'.

30 Analogs of G which are modified in the base portion to form in an

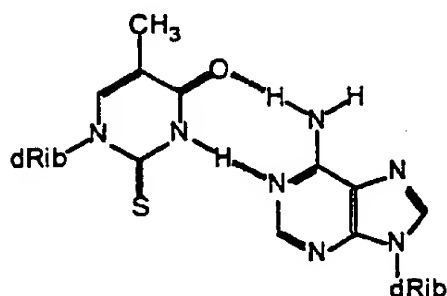
1 ODN-to-nucleic acid or ODN-to-ODN interaction a stable hydrogen
2 bonded pair with C, but not with C' are designated G'. Analogs of C
3 which are modified in the base portion to form in an ODN-to-nucleic
4 acid or ODN-to-ODN interaction a stable hydrogen bonded pair with
5 G, but not with G' are designated C'. The foregoing conditions are
6 satisfied when each of the A', T', G' and C' nucleotides (collectively the
7 modified SBC nucleotides) form, in an ODN-to-nucleic acid or
8 ODN-to-ODN interaction, two or more hydrogen bonds with their
9 natural partner, but only one or no hydrogen bonds with their modified
10 SBC nucleotide partner. This is illustrated by Formulas 1a, 1b, 2a, 2b,
11 3a, 3b, 4a and 4b where the hydrogen bonding between natural A-T (or
12 A-U in case of RNA) and G-C pairs, and hydrogen bonding between
13 exemplary A'-T, T'-A, G'-C, C'-G, A'-T' and G'-C' pairs are illustrated.



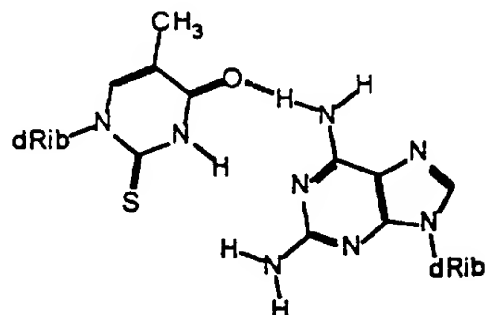
21 T : A 1a



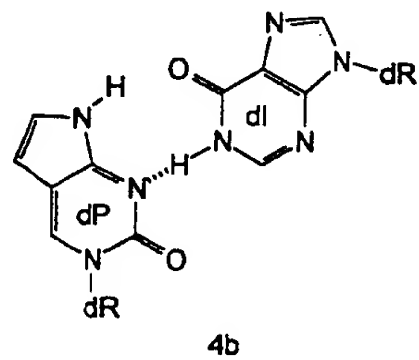
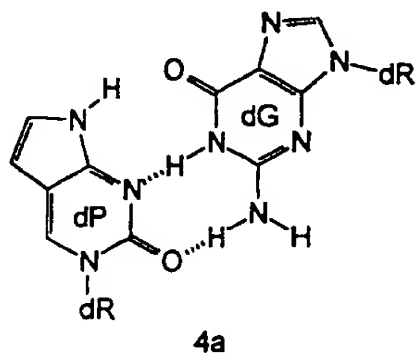
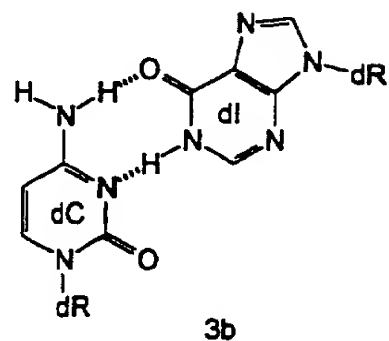
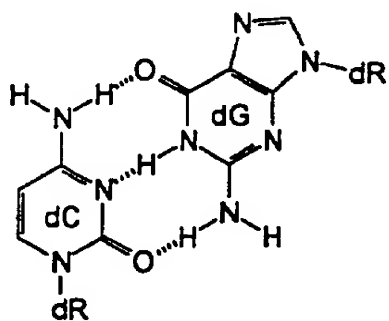
21 T : 2-amA 1b



28 2-sT : A 2a



28 2-sT : 2-amA 2b



1 A sufficient number of the modified SBC nucleotides are
2 incorporated such that complementary positions in both SBC ODNs are
3 modified into a matched pair of SBC ODNs of the present invention so
4 that the pair of the matched set does not form a stable hybrid; in other
5 words under physiological conditions it has a melting temperature of
6 approximately 40°C or less. It is not necessary to replace each natural
7 nucleotide of the ODN with a modified SBC nucleotide in order to
8 accomplish this. Both members of the matched pair are however
9 complementary to a target sequence in double stranded or duplex
10 nucleic acid, where the two strands or parts of the target duplex are
11 themselves complementary or substantially complementary to one
12 another. As it is described in more detail below, an important use of
13 the SBC ODNs of the present invention is hybridization with secondary
14 structure of mRNA wherein the mRNA itself forms a duplex, such as in
15 hairpin loops. It is known that secondary structure of mRNA and
16 ribosomal RNA do not have two strands in the strict sense of that term.
17 Nevertheless, unless the context otherwise indicates, in the present
18 description the terminology "two strands" of double stranded nucleic
19 acids also refers to the two complementary portions of duplex mRNA
20 or of duplex ribosomal RNA as well. The general concept of double
21 stranded DNA and of secondary structure in mRNA and ribosomal
22 RNA is covered in this description by the term "duplex nucleic acid".
23 The term "RNA" can apply to any functional RNA in living organisms,
24 such as messenger, transfer, ribosomal, small nuclear, guide, genomic,
25 etc. RNA.

26 Generally speaking, the SBC ODNs of the present invention
27 include, in addition to the modified SBC nucleotides, the naturally
28 occurring nucleotides, and may also include some other minor naturally
29 occurring or chemically modified nucleotides, as long as such
30 modifications do not interfere significantly with the complementary

1 binding ability of the ODN, as discussed above. Certain important
2 embodiments of the SBC ODNs of the present invention include
3 reporter groups and or cross linking functions covalently attached to one
4 or more nucleotides of the ODN. These embodiments are described in
5 detail below. The SBC ODNs of the present invention may include
6 pentofuranose moieties other than ribose or 2-deoxyribose, as well as
7 derivatives of ribose and 2-deoxyribose, for example
8 3-amino-2-deoxyribose, 2-fluoro-2-deoxyribose, and 2-Q-C₁₋₆ alkyl or
9 2-Q-allyl ribose, particularly 2-Q-methyl ribose. The glycosidic linkage
10 may be of the α or β configuration, with the β configuration being
11 preferred. The phosphate backbone of the SBC ODNs of the present
12 invention may include phosphorothioate linkages. Moreover,
13 cross-linking agents, reporter groups, lipophilic groups (including
14 cholesterol and related "steroid" derivatives) intercalators, minor groove
15 binders as well as alkyl, hydroxy-alkyl, or amino-alkyl tails can also be
16 attached to the 3'- or 5'- phosphate end of the SBC ODNs.

17 The number of nucleotide building units in the SBC ODNs of the
18 present invention is not critical and is generally speaking, in the range
19 of approximately 5 to 99.

20 A general structure for a preferred class of the modified A
21 analog, A', within the scope of the invention and shown as a
22 3'-phosphate (or phosphorothioate) incorporated into the SBC ODN, is
23 provided by Formulas 5, 6 and 7, wherein

24 X is N or CH;

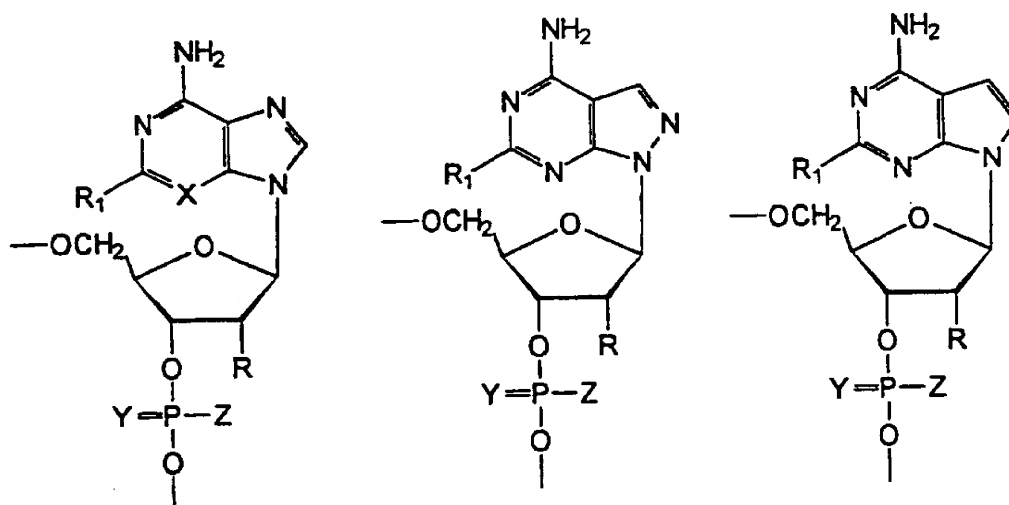
25 Y is O or S;

26 Z is OH or CH₃;

27 R is H, F, or OR₂, where R₂ is C₁₋₆ alkyl or allyl, or H in case of
28 RNA, and

29 R₁ is C₁₋₄ alkyl, C₁₋₄ alkoxy, C₁₋₄ alkylthio, F, or NHR₃, where R₃
30 is H, or C₁₋₄ alkyl, and where the 8 position of the purine, the 3

1 position of the pyrazolopyrimidin or the 5 position of the pyrro-
 2 lopyrimidine optionally serve as point of attachment for a cross-linkig
 3 function, or reporter group as described below. A preferred
 4 embodiment of the SBC nucleotide A' has 2,6-diaminopurine
 5 (2-aminoadenine) as the base, as shown in Formula 1b. The latter
 6 nucleotide is abbreviated as 2-amA or d2-amA as applicable.



Formula 5

Formula 6

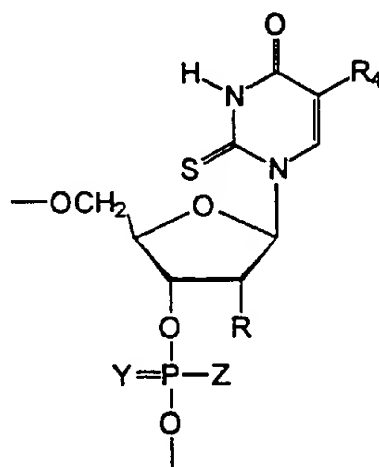
Formula 7

22 A general structure for a preferred class of the modified T
 23 analog, T', within the scope of the invention and shown as a
 24 3'-phosphate (or phosphorothioate) incorporated into the SBC ODN, is
 25 provided by Formula 8, wherein

26 Y, Z and R are defined as above, and

27 R₁ is H, C₁₋₆ alkyl, C₁₋₆ alkenyl, C₁₋₆ alkynyl, or optionally the
 28 5-position of the pyrimidine serves as point of attachment for a
 29 cross-linking function, or a reporter group as described below. A
 30 preferred embodiment of the SBC nucleotide T' has

1 2-thio-4-oxo-5-methylpyrimidine (2-thiothymine) as the base, as shown
 2 in Formula 2b. The latter nucleotide is abbreviated as 2-sT or d 2-sT as
 3 applicable.



18 **Formula 8**

19 A general structure for a preferred class of the modified G
 20 analog, G', within the scope of the invention and shown as a
 21 3'-phosphate (or phosphorothioate) incorporated into the SBC ODN, is
 22 provided by Formulas 9, 10 and 11, wherein

23 R_1 is H, C_{1-4} alkyl, C_{1-4} alkoxy, C_{1-4} alkylthio, F or NHR_3 where R_3
 24 is defined as above,

25 X, Y, Z and R are defined as above, and the 8 position of the
 26 purine, the 3 position of the pyrazolopyrimidine or the 5 position of
 27 the pyrrolopyrimidine optionally serve as point of attachment for a
 28 cross-linking agent, or reporter group as described below. A preferred
 29 embodiment of the SBC nucleotide G' has 6-oxo-purine (hypoxanthine)
 30 as the base, as shown in Formula 3b. The latter nucleotide is

1 abbreviat d as I or dI as applicable.

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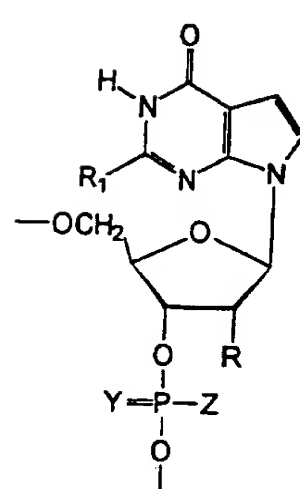
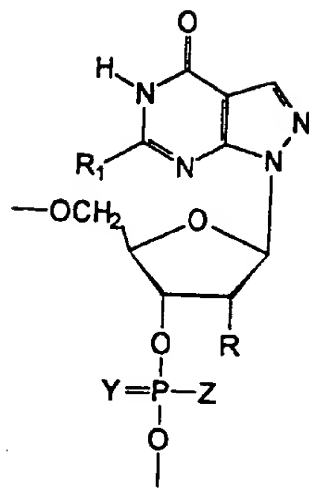
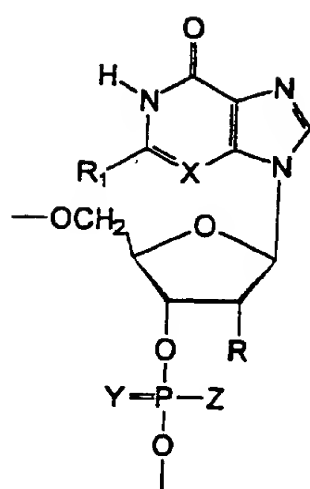
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Formula 9

Formula 10

Formula 11

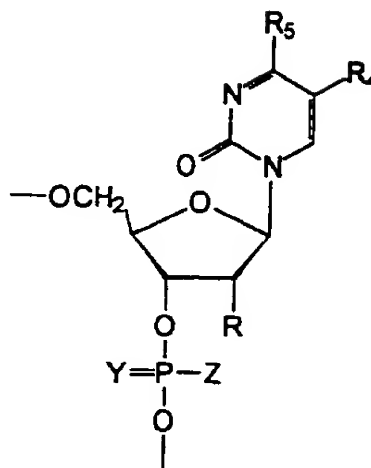
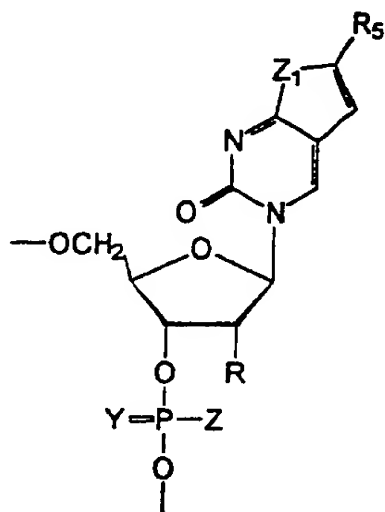
A general structure for a preferred class of the modified C analog, C', within the scope of the invention and shown as a 3'-phosphate (or phosphorothioate) incorporated into the SBC ODN, is provided by Formulas 12 and 13, wherein

Y, Z, R and R₄ are defined as above, or optionally the 5-position of the pyrimidine serves as point of attachment for a cross-linking function, or a reporter group as described below;

Z₁ is O or NH, and

R₃ is H or C₁₋₄ alkyl.

A preferred embodiment of the SBC nucleotide C' has pyrrolo-[2,3-d]pyrimidine-2(3H)-one as the base, as shown in Formula 4b. The latter nucleotide is abbreviated as P or dP as applicable.

**Formula 12****Formula 13**

As noted above, the SBC ODNs of the present invention are utilized in a matched pair where the members of the pair are not covalently linked to one another. In an alternative embodiment the two members of the matched pair may be covalently linked (tethered) to one another with a covalent linkage that does not participate in hybridization and does not prevent hybridization of the two members (each of which is complementary to one target sequence of duplex nucleic acid) to the two strands of the target sequence. Linking groups which are suitable as "tethers" for linking the two SBC ODNs of a matched pair to one another include approximately selected (to avoid hydrogen bonding) nucleotide sequences having approximately 1 to 10 nucleotides. A specific example for the tether would be an ODN moiety having four T's. Alternatively the tethering linkage may comprise the grouping $-\text{[OCH}_2\text{-CH}_2\text{]}_n\text{-O-}$, where n is 1 to 10.

1 **SBC ODNs bearing a cross-linking group**

2 An important class of the SBC ODNs of the present invention
3 bear a cross-linking function or group. The cross-linking function or
4 group may be attached to a nucleotide which is itself an SBC nucleotide
5 (as defined above) or to another type of "natural" or modified
6 nucleotide, and the attachment may be to the heterocyclic base, to the
7 sugar or to a phosphate, preferably a terminal phosphate moiety. The
8 cross linking group or function serves the purpose that after
9 hybridization of the SBC ODN to a target sequence of duplex nucleic
10 acid, the cross-linking function covalently links the SBC ODN to the
11 target. As it will be readily recognized by those skilled in the art,
12 covalent cross-linking increases the efficiency and effectiveness of the
13 SBC ODNs as probes for diagnostic, analytical or other investigative
14 purposes, and also as therapeutic anti-sense and anti-gene agents. A
15 cross-linking group or function may be attached to one or both
16 members of a matched pair of SBC ODNs, and consequently one or
17 both strands of the target sequence may become covalently bonded
18 (alkylated) by this class of SBC ODNs.

19 In light of the foregoing, the cross-linking agents incorporated in
20 the present invention meet the requirements that (1) each cross-linking
21 agent is covalently bonded to a site on the SBC ODN, (2) its length and
22 steric orientation is such that it reaches a suitable reaction site in the
23 target sequence after the SBC ODN is hybridized or complexed with the
24 target and (3) has a reactive group which reacts with a reactive
25 nucleophilic group of the target sequence.

26 In the simplest terms the cross-linking agent itself may
27 conceptually be divided into two groups or moieties, namely the reactive
28 group, which is typically and preferably an electrophilic leaving group
29 (L), and an "arm" (A*) which attaches the leaving group L to the
30 respective site on the SBC ODN. The leaving group L may be chosen

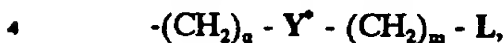
1 from, for example, such groups as chloro, bromo, iodo, $\text{SO}_2\text{R}''$, or
2 $\text{S}^+\text{R}''\text{R}'''$, where each of R'' and R''' is independently C_{1-6} alkyl or aryl or
3 R'' and R''' together form a C_{1-6} alkylene bridge. Chloro, bromo and
4 iodo are preferred. Within these groups haloacetyl groups such as
5 $-\text{COCH}_2\text{I}$, and bifunctional "nitrogen mustards", such as $-\text{N}-[(\text{CH}_2)_2-\text{Cl}]_2$
6 are preferred. The leaving group will be altered by its leaving ability.
7 Depending on the nature and reactivity of the particular leaving group,
8 the group to be used is chosen in each case to give the desired
9 specificity to the irreversibly binding probes or chemotherapeutic agents.

10 Although as noted above the "arm" (or linker arm) A^* may
11 conceptually be regarded as a single entity which covalently bonds the
12 SBC ODN to the leaving group L, and maintains the leaving group L at
13 a desired distance and steric position relative to the SBC ODN, in
14 practice the "arm" A^* may be constructed in a synthetic scheme where a
15 bifunctional molecule is covalently linked to the SBC ODN (for example
16 by a phosphate ester bond to the 3' or 5' terminus, or by a
17 carbon-to-carbon bond to a heterocyclic base) through its first
18 functionality, and is also covalently linked through its second
19 functionality (for example an amine) to a "hydrocarbyl bridge" (alkyl
20 bridge, alkylaryl bridge or aryl bridge, or the like) which, in turn, carries
21 the leaving group.

22 A general formula of the cross linking function is thus $-\text{A}^*-\text{L}$, or
23 $-\text{A}^*-\text{L}_2$, where L is the above defined leaving group and A^* is a moiety
24 that is covalently linked to the SBC ODN. The A^* "arm" moiety itself
25 should be unreactive (other than through the leaving group L) under
26 the conditions of hybridization of the SBC ODN with the target nucleic
27 acid sequence, and should maintain the leaving group L in a desired
28 steric position and distance from the desired site of reaction such as an
29 N-7 position of a guanosine residue in the target sequence. Generally
30 speaking, the length of the A^* group should be equivalent to the length

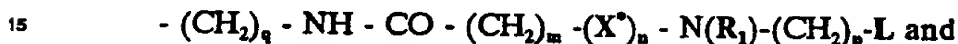
1 of a normal alkyl chain of approximately 2 to 50 carbons.

2 An exemplary more specific formula for a class of preferred
3 embodiments of the cross-linking function is



5 where L is the leaving group, defined above, each of m and q is
6 independently 0 to 8, inclusive, and where Y* is defined as a "functional
7 linking group". A "functional linking group" is a group that has two
8 functionalities, for example -NH₂ and -OH, or -COOH and -OH, or
9 -COOH and -NH₂, which are capable of linking the (CH₂)_q and (CH₂)_m
10 bridges. An acetylenic terminus (HC≡C-) is also a suitable functionality
11 as a precursor for Y*, because it can be coupled to certain heterocycles
12 and thereafter hydrogenated, as described below.

13 Other exemplary and more specific formulas for a class of
14 preferred embodiments of the cross-linking function are



17 where q, m and L are defined as above, q' is 3 to 7 inclusive, q" is
18 1 to 7 inclusive, X* is phenyl or simple substituted phenyl (such as
19 chloro, bromo, lower alkyl or lower alkoxy substituted phenyl), n is 0 or
20 1, p is an integer from 1 to 6, and R₁ is H, lower alkyl or (CH₂)_p-L.
21 Preferably p is 2. Those skilled in the art will recognize that the
22 structure - N(R₁)-(CH₂)₂-L describes a "nitrogen mustard", which is a
23 class of potent alkylating agents. Particularly preferred within this class
24 of SBC ODNs of the invention are those where the cross-linking agent
25 includes the functionality - N(R₁)-(CH₂)₂-L where L is halogen,
26 preferably chlorine; and even more preferred within this class are those
27 modified SBC ODNs where the cross linking agent includes the
28 grouping - N-[(CH₂)₂-L]₂ (a "bifunctional" N-mustard).

29 A particularly preferred partial structure of the cross linking
30 agent includes the grouping

1 $-\text{CO} - (\text{CH}_2)_3 - \text{C}_6\text{H}_4 - \text{N} - [(\text{CH}_2)_2\text{Cl}]_2$.

2 In a particularly preferred embodiment the just-noted cross-linking
3 group is attached to an η -hexylamine bearing tail at the 5' and 3' ends
4 of the SBC ODN in accordance with the following structure:

5 $\text{R}'\text{-O}-(\text{CH}_2)_6\text{-NH} - \text{CO} - (\text{CH}_2)_3 - \text{C}_6\text{H}_4 - \text{N} - [(\text{CH}_2)_2\text{Cl}]_2$

6 where R' signifies the terminal 5' or 3'-phosphate group of the SBC
7 ODN.

8 Other examples for the A*-L group, particularly when attached to
9 a heterocyclic base in the oligonucleotide (such as to the 5-position of
10 2'-deoxyuridine) are 3-iodoacetamidopropyl,
11 3-(4-bromobutyramido)propyl, 4-iodoacetamidobutyl and
12 4-(4-bromobutyramido)butyl groups.

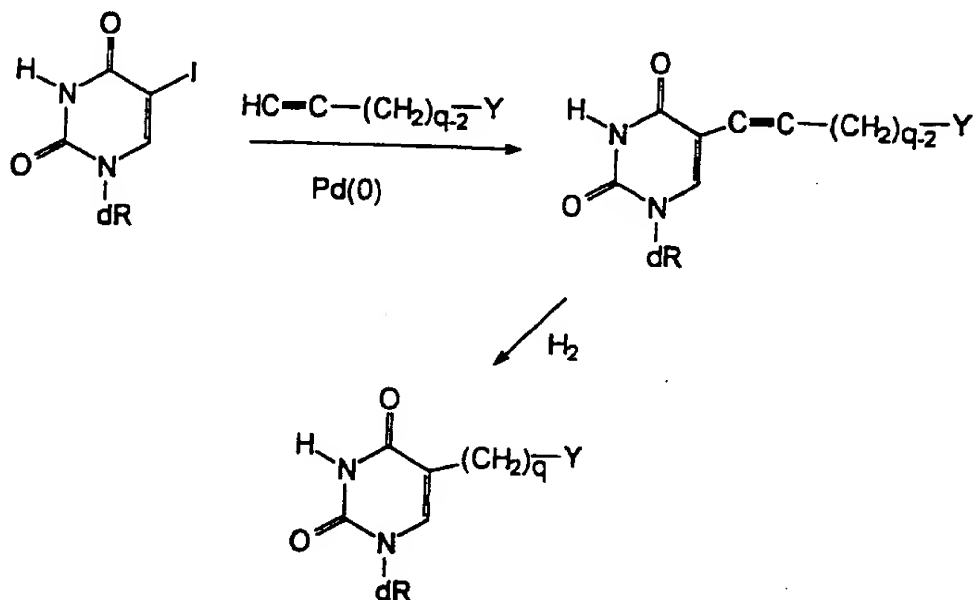
13 In accordance with other preferred embodiments, the
14 cross-linking functionality is covalently linked to the heterocyclic base,
15 for example to the uracil moiety of a 2'-deoxyuridylic acid building
16 block of the SBC ODN. The linkage can occur through the
17 intermediacy of an amino group, that is, the "arm-leaving group
18 combination" (A*-L) may be attached to a 5-amino-2'-deoxyuridylic acid
19 building unit of the SBC ODN. In still other preferred embodiments
20 the "arm-leaving group combination" (A*-L) is attached to the 5-position
21 of the 2'-deoxyuridylic acid building unit of the SBC ODN by a
22 carbon-to-carbon bond. Generally speaking,
23 5-substituted-2'-deoxyuridines can be obtained by an adaptation of the
24 general procedure of Robins et al. (Can. J. Chem., 60:554 (1982); J.
25 Org. Chem., 48:1854 (1983)), as shown in Reaction Scheme 1. In
26 accordance with this adaptation, the palladium-mediated coupling of a
27 substituted 1-alkyne to 5-iodo-2'-deoxyuridine gives an
28 acetylene-coupled product. The acetylenic dUrd analog is reduced, with
29 Raney nickel for example, to give the saturated compound, which is
30 then used for direct conversion to a reagent for use on an automated

1 DNA synthesizer, as described below. In Reaction Scheme 1, q is
2 defined as above, and Y' is either Y* (as defined above) or is a suitable
3 protected derivative of Y*. Y' can also be defined as a group which
4 terminates in a suitably protected nucleophilic function, such as a
5 protected amine. Examples of reagents which can be coupled to
6 5-iodo-2'-deoxyuridine in accordance with this scheme are
7 $\text{HC}\equiv\text{CCH}_2\text{OCH}_2\text{CH}_2\text{N}(\text{CO})_2\text{C}_6\text{H}_4$ (phtalimidoethoxypropyne),
8 $\text{HC}\equiv\text{CCH}_2\text{OCH}_2\text{CH}_2\text{NHCOCF}_3$ (trifluoroacetamidoethoxypropyne),
9 $\text{HC}\equiv\text{CCH}_2\text{N}(\text{CO})_2\text{C}_6\text{H}_4$ (phtalimidopropyne) and $\text{HC}\equiv\text{CCH}_2\text{NHCOCF}_3$,
10 (trifluoroacetamidopropyne),

11 In these examples the nucleosides which are obtained in this
12 scheme are incorporated into the desired SBC ODN, and the alkylating
13 portion of the cross-linking agent is attached to the terminal amino
14 group of "Y" only after removal of the respective phthalic or
15 trifluoroacetyl blocking groups.

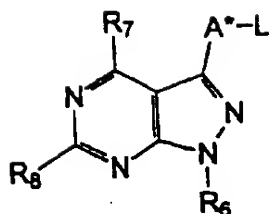
16 Another particularly preferred example of an "arm-leaving group
17 combination" (A*-L) is attachment of a nitrogen-mustard type alkylating
18 agent (or other alkylating agent) to the amino function of a
19 5-(3-aminopropyl)-2'-deoxyuridine building unit of the SBC ODN. The
20 appropriate nucleotide building unit for ODN synthesis which includes
21 the 5-(3-aminopropyl)-2'-deoxyuridine nucleoside moiety can be
22 obtained in analogy to Reaction Scheme 1, and in accordance with the
23 teaching of Meyer et al., J. Am. Chem. Soc. 1989, 111, 8517. In this
24 particularly preferred embodiment the nucleotide having the
25 5-(3-aminopropyl)-2'-deoxyuridine moiety is incorporated into the SBC
26 ODN by routine synthesis, and the cross-linking function is introduced
27 by reacting the SBC ODN with an activated form of a "nitrogen
28 mustard", such as 2,3,5,6-tetrafluorophenyl-4'-[bis(2-chloroethyl)-
29 amino]phenyl- butyrate (Chlorambucil 2,3,5,6-tetrafluorophenyl ester;
30 chlorambucil itself is commercially available).

17



Reaction Scheme 1

Other examples of nucleotides where the crosslinking agent is attached to a heterocyclic base, are 2'-deoxy-4-aminopyrazolo[3,4-d]pyrimidine derivatives. The general structure of these derivatives is shown below in Formula 14. A'-L represents the "arm" and the "leaving group" of the cross-linking functionality, as described above. R_s represents the sugar moiety as described above, and R_1 and R_2 independently are H, OR, SR, NHOR, NH_2 or $\text{NH}(\text{CH}_2)_t\text{NH}_2$, where R is H or C_{1-6} alkyl, t is 0 to 12. These compounds can be made from 3,4-disubstituted and 3,4,6-trisubstituted pyrazolo[3,4-d]pyrimidines, in accordance with the teaching of Kobayashi in Chem. Phar. Bull. 21:941-951 (1973) which is incorporated herein by reference.



Formula 14

Discussing still in general terms the structures of the cross-linking class of SBC ODNs of the present invention, it is noted that examination of double-stranded DNA by ball-and-stick models and high resolution computer graphics indicates that the 7-position of the purines and the 5-position of the pyrimidines lie in the major groove of the B-form duplex of double-stranded nucleic acids. These positions can be substituted with side chains of considerable bulk without interfering with the hybridization properties of the bases. These side arms may be introduced either by derivatization of dThd or dCyd, or by straightforward total synthesis of the heterocyclic base, followed by glycosylation. These modified nucleosides may be converted into the appropriate activated nucleotides for incorporation into oligonucleotides with an automated DNA synthesizer. With the pyrazolo[3,4-*d*]pyrimidines, which are analogs of adenine, the crosslinking arm is attached at the 3-position, which is equivalent to the 7-position of purine.

The crosslinking side chain (arm = A^{*}) should be of sufficient length to reach across the major groove from a purine 7- or 8-position, pyrimidine 5-position, pyrrolopyrimidine 5-position or pyrazolopyrimidine 3-position and react with the N-7 of a purine (preferably guanine) located above (on the oligomer 3'-side) the base pair containing the modified analog.

1 The crosslinking side chain (arm = A*) holds the functional group away
2 from the base when the base is paired with another within the
3 double-stranded complex. As noted above, broadly the arm A* should
4 be equivalent in length to a normal alkyl chain of 2 to 50 carbons.
5 Preferably, the arms include alkylene groups of 1 to 12 carbon atoms,
6 alkenylene groups of 2 to 12 carbon atoms and 1 or 2 olefinic bonds,
7 alkynylene groups of 2 to 12 carbon atoms and 1 or 2 acetylenic bonds,
8 or such groups substituted at a terminal point with nucleophilic groups
9 such as oxy, thio, amino or chemically blocked derivatives thereof (e.g.,
10 trifluoroacetamido, phthalimido, CONR', NR'CO, and SO₂NR', where
11 R' = H or C₁₋₆alkyl). Such functionalities, including aliphatic or
12 aromatic amines, exhibit nucleophilic properties and are capable of
13 serving as a point of attachment to such groups as

14 - (CH₂)_m - L,
15 - CO - (CH₂)_m - (X*)_n - N(R₁) - (CH₂)_p - L, and
16 - CO - CH₂ - L

17 which are described above as components of exemplary cross-linking
18 functional groups.

19 After the nucleoside or nucleotide unit which carries the
20 crosslinking functionality A*-L, or a suitable precursor thereof, (such as
21 the - (CH₂)_q - NH₂ or - (CH₂)_q - Y* group, where Y* terminates with a
22 nucleophilic group such as NH₂) is prepared, further preparation of the
23 modified oligonucleotides of the present invention can proceed in
24 accordance with state-of-the-art. Thus, to prepare oligonucleotides,
25 protective groups are introduced onto the nucleosides or nucleotides
26 and the compounds are activated for use in the synthesis of
27 oligonucleotides. The conversion to protected, activated forms follows
28 the procedures as described for 2'-deoxynucleosides in detail in several
29 reviews. See, Sonveaux, Bioorganic Chemistry, 14:274-325 (1986);
30 Jones, in "Oligonucleotide Synthesis, a Practical Approach", M.J. Gait,

1 Ed., IRL Press, p. 23-34 (1984).

2 The activated nucleotides are incorporated into oligonucleotides
3 in a manner analogous to that for DNA and RNA nucleotides, in that
4 the correct nucleotides will be sequentially linked to form a chain of
5 nucleotides which is complementary to a sequence of nucleotides in
6 target DNA or RNA. The nucleotides may be incorporated either
7 enzymatically or via chemical synthesis. The nucleotides may be
8 converted to their 5'-Q-dimethoxytrityl-3'-(N,N-diisopropyl)-
9 phosphoramidite cyanoethyl ester derivatives, and incorporated into
10 synthetic oligonucleotides following the procedures in "Oligonucleotide
11 Synthesis: A Practical Approach", supra. The N-protecting groups are
12 then removed, along with the other oligonucleotide blocking groups, by
13 post-synthesis aminolysis, by procedures generally known in the art.

14 In a preferred embodiment, the activated nucleotides may be
15 used directly on an automated DNA synthesizer according to the
16 procedures and instructions of the particular synthesizer employed. The
17 oligonucleotides may be prepared on the synthesizer using the standard
18 commercial phosphoramidite or H-phosphonate chemistries. The
19 foregoing description for preparing the SBC ODNs of the invention
20 applies not only to the SBC ODNs which bear one or more cross linking
21 agents, but also generally to all SBC ODNs of the invention. However,
22 as it is described in detail below, 2-thiothymine containing SBC
23 nucleotides (T' analogs) are more sensitive to treatment with ammonia
24 (or other nucleophiles) than other generally used components for
25 sequential ODN synthesis on an automatic synthesizer. The preferred
26 methods for incorporating these components into the SBC ODNs of the
27 invention, and other chemical processes which differ from the normally
28 routine processes of automatic ODN synthesis, are described below.

29 A moiety containing the leaving group, such as a haloacyl group
30 ($\text{CO-CH}_2\text{-L}$ where L is halogen for example I) or $-\text{CO}-(\text{CH}_2)_m-(\text{X}^*)_n-$

1 N(R₁)-(CH₂)_p-L group (even more preferably a
2 CO-(CH₂)₃-C₆H₄-N-[CH₂CH₂Cl]₂) may be added to the aminoalkyl or
3 like groups (-CH₂)_q-Y*) following incorporation into oligonucleotides
4 and removal of any blocking groups. For example, addition of an
5 α-haloacetamide may be verified by a changed mobility of the modified
6 compound on HPLC, corresponding to the removal of the positive
7 charge of the amino group, and by subsequent readdition of a positive
8 charge by reaction with 2-aminoethanethiol to give a derivative with
9 reverse phase HPLC mobility similar to the original
10 aminoalkyl-oligonucleotide.

11 In the situations where the cross linking agent (A*-L moiety) is
12 attached to the 3' or 5' terminus of the oligonucleotide, for example by
13 an alkylamine linkage of the formula -(CH₂)_q-Y* (Y* terminating in an
14 amine), the oligonucleotide synthesis may be performed to first yield the
15 oligonucleotide with said aminoalkyl tail, to which then an alkylating
16 moiety, such as the above-noted haloacylgroup (CO-CH₂-L) or - CO -
17 (CH₂)_m - (X*)_n - N(R₁)-(CH₂)_p-L is introduced.

18 **SBC ODNs bearing a reporter group, lipophilic group or tail**

19 As is known in the art a "reporter group" can be broadly defined
20 as a group that is incorporated in, or is attached to an ODN and which
21 renders detection or isolation of the ODN possible by application of
22 some analytical, physical, chemical or biochemical method. Generally
23 speaking reporter groups are attached to ODNs when the ODNs are
24 used as probes. In terms of attaching reporter groups to ODNs in the
25 general sense, the art is well developed and is recited here only in a
26 summary fashion. The SBC ODNs of the present invention having a
27 reporter group (such as a radioactive label) attached, can be utilized
28 substantially in accordance with state-of-the-art hybridization
29 technology, to detect specific target sequences in duplex regions of
30 nucleic acids. The advantage of the SBC ODNs of the present

1 invention, as compared to the prior art, is that the SBC ODN of the
2 present invention can effectively invade and bind to the duplex nucleic
3 acid sequence.

4 Thus, probes may be labeled by any one of several methods
5 typically used in the art. A common method of detection is the use of
6 autoradiography with ^3H , ^{125}I , ^{35}S , ^{14}C , or ^{32}P labeled probes or the like.
7 Other reporter groups include ligands which bind to antibodies labeled
8 with fluorophores, chemiluminescent agents, and enzymes.
9 Alternatively, probes can be conjugated directly with labels such as
10 fluorophores, chemiluminescent agents, enzymes and enzyme substrates.
11 Alternatively, the same components may be indirectly bonded through a
12 ligand-antiligand complex, such as antibodies reactive with a ligand
13 conjugated with label. The choice of label depends on sensitivity
14 required, ease of conjugation with the probe, stability requirements, and
15 available instrumentation.

16 The choice of label dictates the manner in which the label is
17 incorporated into the probe. Radioactive probes are typically made
18 using commercially available nucleotides containing the desired
19 radioactive isotope. The radioactive nucleotides can be incorporated
20 into probes, for example, by using DNA synthesizers, by
21 nick-translation, by tailing of radioactive bases in the 3' end of probes
22 with terminal transferase or the 5'-end with a polynucleotide kinase.

23 Non-radioactive probes can be labeled directly with a signal (e.g.,
24 fluorophore, chemiluminescent agent or enzyme) or labeled indirectly by
25 conjugation with a ligand. For example, a ligand molecule is covalently
26 bound to the probe. This ligand then binds to a receptor molecule
27 which is either inherently detectable or covalently bound to a detectable
28 signal, such as an enzyme or photoreactive compound. Ligands and
29 antiligands may be varied widely. When a ligand has a natural
30 "antiligand", namely ligands such as biotin, thyroxine, and cortisol, it can
31 be used in conjunction with its labeled, naturally occurring antiligand.

1 Alternatively, any haptenic or antigenic compound can be used in
2 combination with a suitably labeled antibody. A preferred labeling
3 method utilizes biotin-labeled analogs of oligonucleotides, as disclosed
4 in Langer et al., Proc. Natl. Acad. Sci. USA 78:6633-6637 (1981), which
5 is incorporated herein by reference.

6 Enzymes of interest as reporter groups will primarily be
7 hydrolases, particularly phosphatases, esterases, ureases and
8 glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent
9 compounds include fluorescein and its derivatives, rhodamine and its
10 derivatives, dansyl, umbelliferone, rare earths, etc. Chemiluminescers
11 include luciferin, acridinium esters and 2,3-dihydrophthalazinediones,
12 e.g., luminol. A further description of reporter groups and specific
13 examples thereof can be found in United States Patent No. 5,419,966,
14 the specification of which is expressly incorporated herein by reference.

15 The specific hybridization conditions are not critical and will vary
16 in accordance with the investigator's preferences and needs. The
17 particular hybridization technique is not essential to the invention.
18 Hybridization techniques are generally described in "Nucleic Acid
19 Hybridization, A Practical Approach", Hames and Higgins, Eds., IRL
20 Press, 1985; Gall and Pardue, Proc. Natl. Acad. Sci., U.S.A., 63:378-383
21 (1969); and John et al., Nature, 223:582-587 (1969). As improvements
22 are made in hybridization techniques, they can readily be applied.

23 The amount of labeled probe which is present in the hybridization
24 solution may vary widely. Generally, substantial excess of probe over
25 the stoichiometric amount of the target duplex nucleic acid will be em-
26 ployed to enhance the rate of binding of the probe to the target
27 sequence.

28 After hybridization at a temperature and time period appropriate
29 for the particular hybridization solution used, the glass, plastic, or filter
30 support to which the probe-target hybrid is attached is introduced into a
31 wash solution typically containing similar reagents as provided in the

1 hybridization solution. Either the hybridization or the wash medium
2 can be stringent. After appropriate stringent washing, the correct
3 hybridization complex may now be detected in accordance with the
4 nature of the label.

5 The probe may be conjugated directly with the label. For
6 example, where the label is radioactive, the support surface with
7 associated hybridization complex substrate is exposed to X-ray film.
8 Where the label is fluorescent, the sample is detected by first irradiating
9 it with light of a particular wavelength. The sample absorbs this light
10 and then emits light of a different wavelength which is picked up by a
11 detector ("Physical Biochemistry", Freifelder, D., W. H. Freeman & Co.,
12 1982, pp. 537-542). Where the label is an enzyme, the sample is
13 detected by incubation with an appropriate substrate for the enzyme.
14 The signal generated may be a colored precipitate, a colored or
15 fluorescent soluble material, or photons generated by bioluminescence
16 or chemiluminescence. The preferred label for dipstick assays generates
17 a colored precipitate to indicate a positive reading. For example,
18 alkaline phosphatase will dephosphorylate indoxyl phosphate which then
19 will participate in a reduction reaction to convert tetrazolium salts to
20 highly colored and insoluble formazans.

21 Detection of a hybridization complex may require the binding of
22 a signal generating complex to a duplex of target and probe
23 polynucleotides or nucleic acids. Typically, such binding occurs through
24 ligand and antiligand interactions as between a ligand-conjugated probe
25 and an antiligand conjugated with a signal.

26 The label may also allow indirect detection of the hybridization
27 complex. For example, where the label is a hapten or antigen, the
28 sample can be detected by using antibodies. In these systems, a signal is
29 generated by attaching fluorescent or enzyme molecules to the
30 antibodies or in some cases, by attachment to a radioactive label.
31 (Tijssen, P., "Practice and Theory of Enzyme Immunoassays, Laboratory

1 Techniques in Biochemistry and Molecular Biology", Burdon, R.H., van
2 Knippenberg, P.H., Eds., Elsevier, 1985, pp. 9-20).

3 The amount of labeled probe present in the hybridization solution
4 may vary widely, depending upon the nature of the label, the amount of
5 the labeled probe that can reasonably bind to the cellular target nucleic
6 acids, and the precise stringency of the hybridization medium and/or
7 wash medium. Generally, substantial probe excesses over the
8 stoichiometric amount of the target will be employed to enhance the
9 rate of binding of the probe to the target nucleic acids.

10 This aspect of the invention is also directed to a method for
11 identifying target duplex nucleic acid sequences, which method
12 comprises utilizing an SBC ODN probe including a label as described
13 above.

14 In one embodiment, the method comprises the steps of:

- 15 (a) preparing nucleic acids in the sample to be tested;
- 16 (b) hybridizing to the target nucleic acids an SBC ODN
17 probe wherein the SBC ODN is a matched pair where each ODN of the
18 pair is complementary to one of the two complementary strands of the
19 target nucleic acid sequence;
- 20 (c) washing the sample to remove unbound probe;
- 21 (d) incubating the sample with detecting agents; and
- 22 (e) inspecting the sample.

23 The above method may be conducted following procedures well
24 known in the art.

25 The SBC ODNs of the present invention may also incorporate
26 lipophilic groups, especially as a "tail" moiety attached to the 3' or 5'
27 phosphate end of the ODN, and related tails, such as aminoalkyl groups
28 (having approximately 3 to 20 carbons), or hydroxyalkyl groups (having
29 approximately 3 to 20 carbons). As is known in the art, lipophilic
30 groups are groups which due to their hydrophobic nature substantially

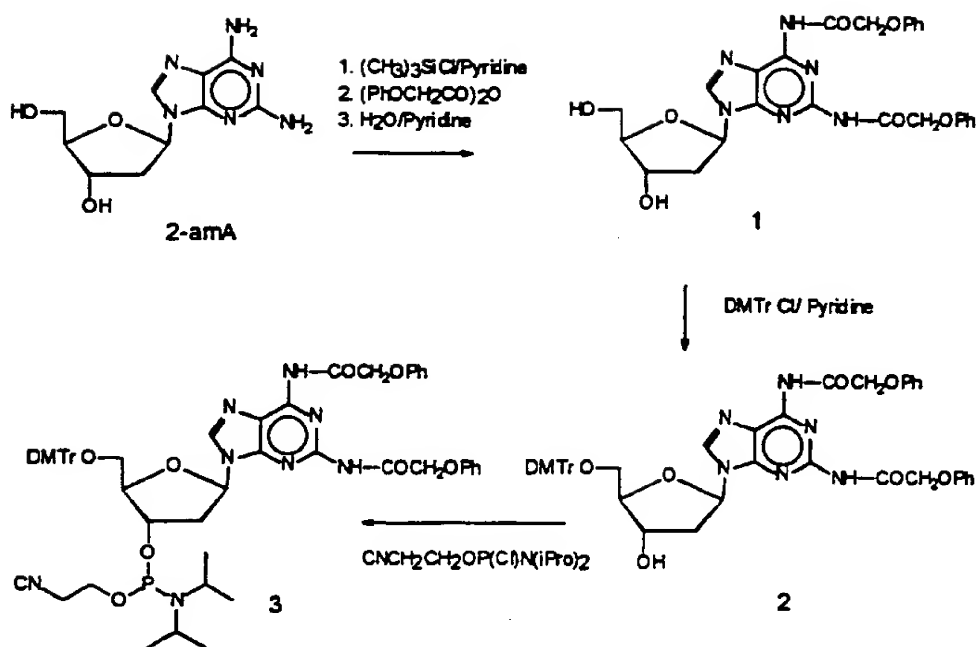
1 increase lipid solubility of a compound. Examples for lipophilic groups,
2 are long chain (3 to 20 carbon alkyl, cycloalkyl groups, and compounds
3 having a "steroid" skeleton such as cholesterol, cholic acid, progesterone
4 and estradiol. Further examples of lipophilic groups are menthol and
5 retinoic acid or analogs of retinoic acid. Synthetic methods suitable for
6 attaching lipophilic and other tail moieties to the 3' or 5' end of the
7 SBC ODNs of the present invention are described in United States
8 Patent No. 5,419,966 the specification of which is expressly incorporated
9 herein.

10 Preparation of the SBC ODNs of the Invention

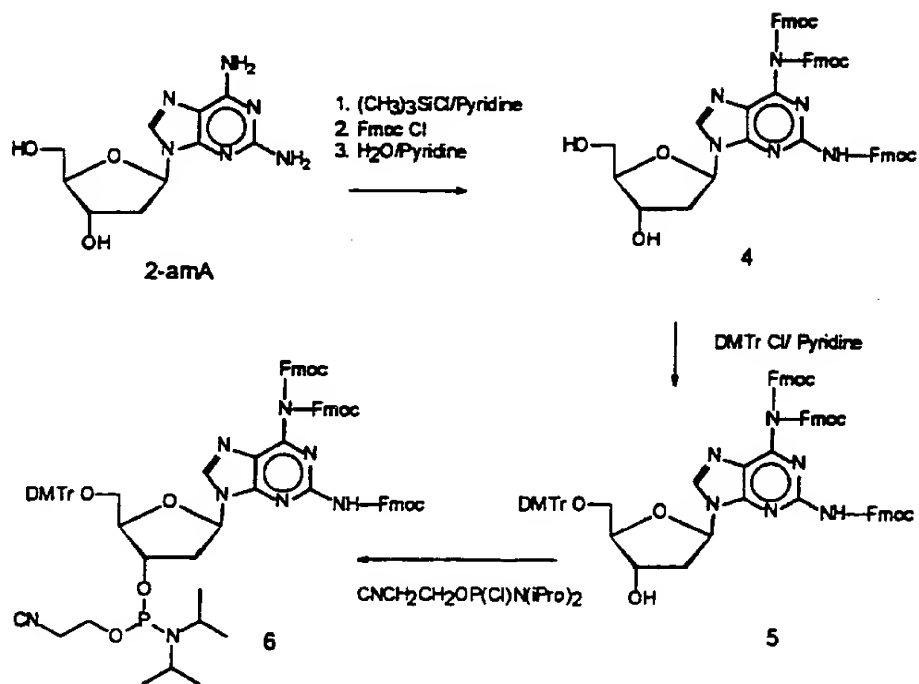
11 The nucleosides and nucleotides shown as components of the
12 SBC ODNs of the present invention can be made by procedures known
13 in the chemical literature. Oligonucleotide synthesis on an automatic
14 synthesizer is generally described above in connection with the
15 description of SBC ODNs containing a cross-linking functionality. A
16 more detailed description of ODN synthesis with an automatic
17 synthesizer utilizing a modified solid support, which is used in the
18 currently preferred method for preparing the SBC ODNs of the
19 invention, is described in United States Patent No. 5,419,966.

20 A modification of the standard "phosphoramidite" ODN synthesis
21 procedure is used, however, when 2-thiothymine containing SBC ODNs
22 are prepared because this heterocycle is more base labile than the
23 natural base heterocycles of nucleic acids. Therefore, when this
24 nucleotide is involved, milder treatment with ammonia is required in the
25 step of removing blocking groups from the exocyclic amino groups of
26 the nucleotide components and to remove the SBC ODN from the solid
27 support. The modified procedures for preparing the suitably protected
28 "phosphoramidite" reagents of 2,6-diaminopurine-2'-deoxyribofuranoside
29 (Compounds 3 and 6) for nucleic acid synthesis are illustrated, in the
30 alternative, in Reaction Schemes 2 and 3. As it can be seen in Reaction

1 Scheme 2, the phenoxyacetyl blocking group is attached to the exocyclic
2 amino groups, whereas in Reaction Scheme 3 the
3 9-fluorenylmethoxycarbonyl (Fmoc) protecting group is used.
4 N-phenoxyacetyl protected 2'-deoxyguanosine and 2'-deoxycytidine
5 3'-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidites are available
6 commercially from BioGenex, Alameda, California.
7 5'-O-Dimethoxytrityl-2-thiothymidine-3'-O-(2-cyanoethyl)-N,N-diisoprop
8 ylphosphoramidite) can be obtained in accordance with the known
9 literature procedure of Connolly et al. (1989) Nucleic Acids Res. 17,
10 4957-4974. 2,6-Diaminopurine-2'-deoxyribose (the starting material in
11 Reaction Schemes 2 and 3) can be obtained in accordance with the
12 known literature procedure of Fathi et al. Tetrahedron Lett. 31,
13 319-322.

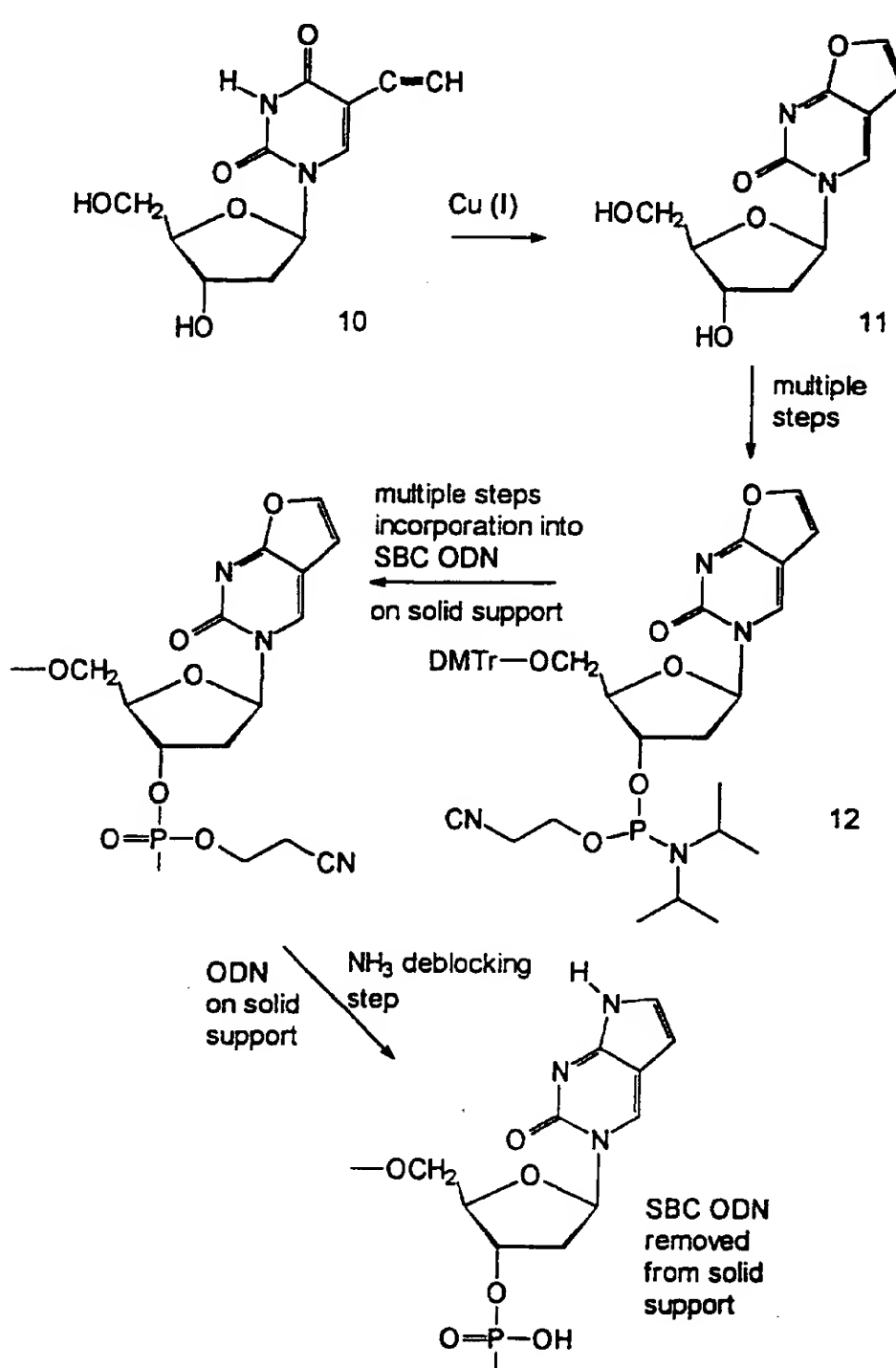


Reaction Scheme 2



1 The nucleotide moiety shown in Formula 4b is obtained by the
2 method illustrated in Reaction Scheme 4 which substantially follows
3 known chemical literature. First the "furan" analog deoxyribofuranoside,
4 namely 3-(2'-deoxy-β-D-ribofuranosyl)furano-[2,3-d]pyrimidine-
5 6(5H)-one (Compound 11) is synthesized by copper (I)-catalyzed
6 cyclization from the known antiviral nucleoside
7 5-ethynyl-2'-deoxyuridine (Compound 10), substantially as in the
8 literature procedure of Robins et al. J. Org. Chem. 1983, 48, 1854. This
9 compound is dimethoxytritylated and converted into the corresponding
10 cyanoethoxy phosphoramidite (Compound 12) suitable as a reagent for
11 ODN synthesis, substantially by conventional literature methods (see
12 Sinha et al. Nucleic Acids Research. 1984, 12, 4539). The SBC ODNs
13 of the present invention are then constructed on a solid support. The
14 final step of treating the SBC ODN with ammonia to remove protecting
15 groups, converts the furano-[2,3-d]pyrimidine-6(5H)-one base into the
16 pyrrolo-[2,3-d]pyrimidine-6(5H)-one base shown in Formula 4b. The
17 Connolly et al. Nucleic Acids Res. 1989 17, 4957-4974, Fathi et al.
18 Tetrahedron Lett. 1990 31, 319-322, Robins et al. J. Or. Chem. 1983, 48,
19 1854 and Sinha et al. Nucleic Acid Research. 1984, 12, 4539 publications
20 are expressly incorporated herein by reference.

31



Reaction Scheme 4

Use of the SBC ODNs of the invention and evidence of sequence specific selective binding ability

Several oligonucleotides were prepared containing dI for dG, dP for dC, or containing d2-sT for dT and d2-amA for dA. The hybridization properties of these ODNs were studied by determining the melting temperature of the hybrids (under substantially physiological conditions) and by non-denaturing polyacrylamide gel electrophoresis (hereinafter PAGE) analysis. These measurements confirmed that each of the SBC ODNs forms a stable hybrid with the natural complementary or (substantially complementary) ODN, but not with the complementary SBC ODN. Thus, members of a matched pair of SBC ODNs were found to form stable hybrids with their respective natural complementary targets, but not with each other. Table 1 below indicates the melting temperatures observed under the conditions indicated in the table, and also the calculated decrease (drop) in melting temperature per modified base pair.

Table 1

Table 1. T_m Values for Native and Modified ODNs with dI and dP

Watson: 5' XTY AXA AXY ATX YYA YYA XXY AAY YAY X 3'
Crick: 3' YAX TYT TYX TAY XXT XXT YYX TTX XTX Y 5'

Hybrid	Watson		Crick		T _m (°C) ^a	T _m Drop per Modified Base Pair
	X	Y	X	Y		
I	C	G	C	G	75.6	0
II	P	I	C	G	48.2	1.61
III	C	G	P	I	57.2	1.08
IV	P	I	P	I	20.2	3.26

a: 10mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 50 mM NaCl, 10 mM MgCl₂

1 In Table 1 the 28-mer ODN is a sequence taken from pBR 322
2 plasmid. Hybrid 1 is formed from complementary
3 oligodeoxynucleotides wherein X and Y are natural dC and dG residues
4 in both ODNs. Thus, Hybrid 1 provides a reference, to which other
5 hybrids formed of modified SBC ODNs can be compared. The pair of
6 SBC ODNs shown as Hybrid IV in Table 1 comprises two 28-mer
7 sequences where each of the natural dG and dC nucleotides is replaced
8 with dI and dP, respectively. Hybrid IV is unstable with a melting
9 temperature of 20.2° C. Nevertheless, each member of this pair forms a
10 stable hybrid with its natural complement, in Hybrids II and III.

11 PAGE analysis also showed that the two members of the matched
12 pair of SBC 28-mers do not hybridize in a stable manner, and that each
13 SBC ODN and its natural complement form a stable hybrid. Moreover,
14 the normal Watson strand showed no preference for the normal Crick
15 strand over the SBC Crick strand because when equimolar amounts of
16 these three strands were mixed simultaneously at room temperature
17 about equal amounts of the duplex Hybrids I and III were formed.
18 Additionally, there was little, if any, strand displacement or strand
19 exchange when pre-formed Hybrid III was incubated with the normal
20 homolog of the SBC strand, or with the Hybrid II. These data
21 demonstrate that the SBC ODNs behave like natural ODNs when
22 hybridized with their unmodified complementary strands, while they do
23 not form stable hybrids with themselves.

24 Table 2 refers to a complementary pair of 20-mer
25 oligodeoxyribonucleotides (ODN V and ODN VI) which are hybridized
26 under substantially physiological conditions (0.2M NaCl, 0.01M
27 Na₂HPO₄, 0.1mM EDTA, pH7.0, ODN concentration = 4×10^{-7} M).
28 The ODNs designated in Table 2 as SBC(V) and SBC(VI) are modified
29 so that each dA and each dT is replaced with the d2amA and d2sT,
30 respectively. The melting temperatures of these pairs are indicated in

1 the Table.

2 **Table 2**

3 ODN V 5'-GTAAGAGAATTATGCAGTGC-3'
 4 ODN VI 3'-CATTCTCTTAATACGTCACG-5'
 5 SBC(V) 5'-G2sT2amA2amAG2amAG2amA2amA2sT2sT2amA2sTGC2-
 6 amAG2sTGC-3'
 7 SBC(VI) 3'-C2amA2sT2sTC2sTC2sT2sT2amA2amA2sT2amACG2s-
 8 TC2amACG-5'

9
 10 **MELTING TEMPERATURE OF HYBRIDS**

11	ODN(V)	ODN(VI)	SBC(V)	SBC(VI)
12				
13	ODN(V)	-	55° C	- 64° C
14				
15	ODN(VI)	55° C	-	65° C -
16				
17	SBC(V)	-	65° C	- 26° C
18				
19	SBC(VI)	64° C	-	26° C -

20
 21 As it can be seen, the ODNs fully modified with the preferred A'
 22 and T' modifications of the present invention exhibit even stronger
 23 binding to the natural complementary ODNs than the binding between
 24 two natural complementary strands. At the same time, the matched
 25 pair of SBC ODNs are nevertheless incapable of forming a stable hybrid
 26 with each other (their melting temperature is 26°C.

27 Additional experiments conducted in accordance with the present
 28 invention, in terms of melting temperature measurements and PAGE
 29 analysis, showed that a matched pair of SBC ODNs complementary to
 30 both strands of a target sequence of double stranded DNA is capable of

1 invading the natural duplex nucleic acid to give a stable 3-armed joint.
2 Analogous paired normal DNA ODNs failed to invade the same target.
3 In case of long double stranded DNA, sequentially hybridizing the
4 paired SBC ODNs to each member of the DNA target and then
5 combining these hybrids results in stable double D loop formation which
6 is stabilized by the bonding between each member of the SBC ODN
7 pair and the corresponding complementary sequence in the target DNA.
8 The resulting three-arm joints between the SBC ODNs and the DNA
9 can be cleaved by resolvase enzymes. Strand invasion and double
10 D-loop formation in long DNA by paired SBC ODNs is catalyzed by
11 recombinase enzymes such as recA. Cleavage of these sites by resolvase
12 will allow restriction of very long DNA, as from genomic DNA or
13 cDNA libraries, at any pre-selected site. Therefore, the SBC ODNs of
14 the invention can be used for gene mapping and like analytical and
15 diagnostic purposes. The SBC ODNs can also be utilized to inhibit or
16 block expression of a target gene, especially when one or preferably
17 both members of the matched pair of SBC ODNs include a cross-linking
18 function. In such a case, after double D loop formation with the target
19 sequence of the gene, both strands of the nucleic acid are covalently
20 linked to the SBC ODN, resulting in effective suppression of the gene.
21 SBC ODNs having cross-linking functions can also be utilized for gene
22 mapping and like diagnostic purposes.

23 Diagnostic and other "probe" like applications of the SBC ODNs
24 of the invention also extend to messenger and ribosomal RNA, because
25 a matched pair of SBC ODNs is able to sequence specifically invade the
26 secondary structure of these duplex ribonucleic acids. Therapeutic use
27 is in the anti-sense field, especially when the SBC ODN includes a
28 cross-linking functionality. It is known in the art that the sequence of
29 ribosomal RNA of bacteria is species specific. Furthermore, detection
30 of this rRNA in DNA probe-based assays is usually hampered by lack of

1 access of the probe to the RNA because of secondary structure.
2 Accordingly, SBC ODNs designed to sequence specifically invade
3 bacterial ribosomal RNA are used, in accordance with the present
4 invention, in diagnostic applications to diagnose bacterial infections in
5 humans and animal species.

6 EXPERIMENTAL SECTION -- SPECIFIC EXAMPLES

7 Synthesis of pyrazolo[3,4-d]pyrimidin nucleotides

8 EXAMPLE 1:

9 6-(Tritylamino)caproic Acid.

10 6-Aminocaproic acid (26 g, 0.2 mole) was dissolved in
11 dichloromethane (200 mL) by the addition of triethylamine (100 mL).
12 Trityl chloride (120 g, 0.45 mole) was added and the solution stirred for
13 36 hours. The resulting solution was extracted with 1N HCl and the
14 organic layer evaporated to dryness. The residue was suspended in
15 2-propanol/1N NaOH (300 mL/100 mL) and refluxed for 3 hours. The
16 solution was evaporated to a thick syrup and added to dichloromethane
17 (500 mL). Water was added and acidified. The phases were separated,
18 and the organic layer dried over sodium sulfate and evaporated to
19 dryness. The residue was suspended in hot 2-propanol, cooled, and
20 filtered to give 43.5 (58%) of 6-(tritylamino)caproic acid, useful as an
21 intermediate compound.

22 EXAMPLE 2:

23 5-(Tritylamino)pentylhydroxymethylenemalononitrile.

24 To a dichloromethane solution of 6-(tritylamino)-caproic acid
25 (20.0 g, 53 mmole) and triethylamine (20 mL) in an ice bath was added
26 dropwise over 30 min isobutylchloroformate (8.3 mL, 64 mmole). After
27 the mixture was stirred for 2 hours in an ice bath, freshly distilled
28 malononitrile (4.2 g, 64 mmole) was added all at once. The solution
29 was stirred for 2 hours in an ice bath and for 2 hours at RT. The
30 dichloromethane solution was washed with ice cold 2N HCl (300 mL)
31 and the biphasic mixture was filtered to remove product that

1 precipitated (13.2 g). The phases were separated and the organic layer
2 dried and evaporated to a thick syrup. The syrup was covered with
3 dichloromethane and on standing deposited fine crystals of product.
4 The crystals were filtered and dried to give 6.3 g for a total yield of 19.5
5 g (87%) of the product, which is useful as an intermediate.

6 **EXAMPLE 3:**

7 **5-(Tritylamino)pentylmethoxymethylenemalononitrile.**

8 A suspension of the malononitrile of Example 2 (13 g, 31 mmole)
9 in ether/dichloromethane (900 mL/100 mL), cooled in an ice bath, was
10 treated with a freshly prepared ethereal solution of diazomethane (from
11 50 mmole of Diazald^R (Aldrich Chemical Company)). The solution was
12 stirred for 6 hours and then neutralized with acetic acid (10 mL). The
13 solution was evaporated to dryness and the residue chromatographed on
14 silica gel using dichloromethane/acetone (4/1) as the eluent. Fractions
15 containing product were pooled and evaporated to a syrup. The syrup
16 was triturated with dichloromethane to induce crystallization. The
17 crystals were filtered and dried to give 8.3 g (61%) of
18 chromatographically pure product, useful as an intermediate compound.

19 **EXAMPLE 4:**

20 **5-Amino-3-[(5-tritylamino)pentyl]pyrazole-4-carbonitrile.**

21 To a methanol solution (100mL) of the product of Example 3
22 (7.0 g, 16 mmole) in an ice bath was added hydrazine monohydrate (7.8
23 mL, 160 mmole) dropwise over 15 min. After stirring for 30 min in an
24 ice bath, the solution was evaporated to dryness. The residue was
25 suspended in cold methanol and filtered to give 7.1 g (100%) of
26 5-amino-3-[(5-tritylamino)pentyl]pyrazole-4-carbonitrile, useful as an
27 intermediate, after drying. An analytical sample was prepared by
28 recrystallization from water.

29 **EXAMPLE 5:**

30 **5-Amino-1-(2-deoxy-3,5-di-O-toluoyl-β-D-erythrop nto-**
31 **furanosyl)-3-[(5-tritylamino)pentyl]pyrazole-4-carbonitril .**

1 An ice cold solution of the carbonitrile from Example 4 (3.5 g, 8
2 mmole) was treated with sodium hydride and stirred for 30 min at
3 0-4°C. 1-Chloro-1,2-dideoxy-3,5-di-O-toluoylribofuranose was added
4 and the solution stirred for 1 hour at 0-4°C. The solution was poured
5 into a saturated solution of sodium bicarbonate and extracted with
6 dichloromethane. The organic layer was dried over sodium sulfate and
7 evaporated to dryness. The residue was flash chromatographed. The
8 organic layer was dried over sodium sulfate and evaporated to dryness.
9 The residue was flash chromatographed on silica gel using toluene/ethyl
10 acetate (5/1) as eluent. Two major products were isolated and
11 identified as the N-1 and N-2 isomers in 57% (3.6 g) and 20% (1.2 g)
12 N-1 and N-2 yields, respectively. Approximately 1 g of a mixture of N-1
13 and N-2 isomers was also collected. Overall yield of glycosylated
14 material was 5.8 g (92%). The N-1 isomer,
15 5-amino-1-(2-deoxy-3,5-di-o-toluoyl-β-D-erythropentofuranosyl)-3-[(5-trit
16 yl-amino)-pentyl]pyrazole-4-carbonitrile, was used without further
17 purification in Example 6.

18 **EXAMPLE 6:**

19 1-(2-Deoxy-β-D-erythropentofuranosyl)-3-[5-(trityl-
20 amino)-pentyl]pyrazolo[3,4-d]pyrimidin-4-amine.

21 To a toluene (100 mL) solution of the pyrazole-4-carbonitrile of
22 Example 5 (3.5 g, 4.4 mmole) was added diethoxymethyl acetate (1.1
23 mL, 6.7 mmole). The solution was kept at 80-90°C for 5 hours and then
24 evaporated to a syrup. The syrup was dissolved in dichloromethane (10
25 mL) and added to ice cold methanolic ammonia (100 mL) in a glass
26 pressure bottle. After two days at RT the contents of the bottle were
27 evaporated to dryness. The residue was dissolved in methanol and
28 adjusted to pH 8 with freshly prepared sodium methoxide to complete
29 the deprotection. After stirring overnight the solution was treated with
30 Dowex^R-50 H+ resin, filtered and evaporated to dryness. The residue
31 was chromatographed on silica gel using acetone/hexane (3/2) as eluent

1 to give 2.0 g (77%) of analytically pure product.

2 **EXAMPLE 7:**

3 1-(2-Deoxy-β-D-erythropentofuranosyl)-3-[5-(trityl-
4 amino)-pentyl]pyrazolo[3,4-d]pyrimidin-4-amine 5'-monophosphate.

5 To an ice cold solution of the pyrazolopyrimidin-4-amine of
6 Example 6 (250 mg, 0.43 mmole) in trimethyl phosphate (5 mL) was
7 added phosphoryl chloride (50 μL) and the solution was kept at 0-4°C.
8 The reaction was monitored by reversed phase HPLC using a linear
9 gradient from 0 to 100% acetonitrile in water over 25 min. After
10 stirring for 5 hours, an additional aliquot of phosphoryl chloride (25 μL)
11 was added and the solution was stirred another 30 min. The solution
12 was poured into 0.1M ammonium bicarbonate and kept in the cold
13 overnight. The solution was then extracted with ether and the aqueous
14 layer evaporated to dryness. The residue was dissolved in water (5 mL)
15 and purified by reversed phase HPLC using a 22mm X 50cm C18
16 column. The column was equilibrated in water and eluted with a
17 gradient of 0 to 100% acetonitrile over 20 min. Fractions containing
18 the desired material were pooled and lyophilized to give 160 mg (56%)
19 of chromatographically pure nucleotide.

20 **EXAMPLE 8:**

21 1-(2-Deoxy-β-D-erythropentofuranosyl)-3-[5-[(6-bio-
22 tinamido)hexanamido]pentyl]pyrazolo[3,4-d]pyrimidin-4-amine
23 5'-monophosphate.

24 An ethanol solution (10 mL) of the nucleotide of Example 7,
25 palladium hydroxide on carbon (50 mg), and cyclohexadiene (1 mL) was
26 refluxed for 3 days, filtered, and evaporated to dryness. The residue
27 was washed with dichloromethane, dissolved in DMF (1.5 mL)
28 containing triethylamine (100 mL), and treated with
29 N-hydroxy-succinimidyl biotinylaminocaproate (50 mg). After stirring
30 overnight an additional amount of N-hydroxysuccinimidyl
31 6-biotinamidocaproate (50 mg) was added and the solution was stirred

1 for 18 hours. The reaction mixture was evaporated to dryness and
2 chromatographed following the procedure in Example 7. Fractions were
3 pooled and lyophilized to give 80 mg of chromatographically pure
4 biotinamido-substituted nucleotide.

5 **EXAMPLE 9:**

6 **1-(2-Deoxy- β -D-erythropentofuranosyl)-3-[5-(6-biotin-**
7 **amido)-hexanamidopentyl]pyrazolo[3,4-d]pyrimidin-4-amine**
8 **5'-triphosphate.**

9 The monophosphate of Example 8 (80 mg, ca. 0.1 mmole) was
10 dissolved in DMF with the addition of triethylamine (14 μ L).
11 Carbonyldiimidazole (81 mg, 0.5 mmole) was added and the solution
12 stirred at RT for 18 hours. The solution was treated with methanol (40
13 μ L), and after stirring for 30 minutes tributylammonium pyrophosphate
14 (0.5 g in 0.5 mL DMF) was added. After stirring for 24 hours another
15 aliquot of tributylammonium pyrophosphate was added and the solution
16 was stirred overnight. The reaction mixture was evaporated to dryness
17 and chromatographed following the procedure in Example 8. Two
18 products were collected and were each separately treated with conc.
19 ammonium hydroxide (1 mL) for 18 hours at 55°C. UV and HPLC
20 analysis indicated that both products were identical after ammonia
21 treatment and were pooled and lyophilized to give 35.2 mg of
22 nucleoside triphosphate.

23 **EXAMPLE 10:**

24 **NICK-TRANSLATION REACTION**

25 The triphosphate of Example 9 was incorporated into pHPV-16
26 using the nick translation protocol of Langer et al. (supra). The probe
27 prepared with the triphosphate of Example 9 was compared with probe
28 prepared using commercially available bio-11-dUTP (Sigma Chemical
29 Co). No significant differences could be observed in both a filter
30 hybridization and in *in situ* smears.

31 More specifically, the procedure involved the following materials

1 and steps

2 Materials:

3 DNase (ICN Biomedicals) - 4 μ g/mL

4 DNA polymerase 1 (U.S. Biochemicals) -

5 8 U/mL

6 pHPV - 16 - 2.16 mg/mL which is a

7 plasmid containing the genomic

8 sequence of human papillomavirus

9 type 16.

10 10X-DP - 1M Tris,pH7.5(20mL); 0.5M

11 OTT(80 mL); 1M MgCl₂(2.8 mL);

12 H₂O(17mL)

13 Nucleotides - Mix A - 2mM each dGTP,

14 dCTP, TTP (Pharmacia)

15 Mix U - 2mM each dGTP, DcTP,

16 dATP

17 Bio-11-dUTP - 1.0 mg/mL (BRL)

18 Bio-12-dAPPTP - 1.0 mg/mL

19 Steps:

20 To an ice cold mixture of 10X-DP (4 mL), pHPV-16 (2 mL),
21 nucleotide mix A (6 mL), Bio-12-dAPPTP (2 mL), and H₂O (20 mL)
22 was added DNase (1 mL) and DNA polymerase 1 (2.4 mL). The
23 reaction mixture was incubated at 16°C for 1 hour. The procedure was
24 repeated using Bio-11-dUTP and nucleotide mix U in place of
25 Bio-12-dAPPTP (comprising the triphosphate of Example 9) and
26 nucleotide mix A.

27 Nucleic acid was isolated by ethanol precipitation and hybridized
28 to pHPV-16 slotted onto nitrocellulose. The hybridized biotinylated
29 probe was visualized by a streptavidin-alkaline phosphatase conjugate
30 with BCIP/NBT substrate. Probe prepared using either biotinylated
31 nucleotide gave identical signals. The probes were also tested in an in

1 situ format on cervical smears and showed no qualitative differences in
2 signal and background.

3 **EXAMPLE 11:**

4 **5-Amino-3-[(5-tritylamino)pentyl]pyrazole-4-carbox- amide.**

5 Following the procedure of Example 2, except that
6 cyanoacetamide is used instead of malononitrile,
7 5-(tritylamino)pentylhydroxymethylecycanoacetamide is prepared from
8 6-(tritylamino)caproic acid. This is then treated with diazomethane to
9 give the methoxy derivative, following the procedures of Example 3,
10 which is then reacted with hydrazine monohydrate, as in Example 4, to
11 give 5-amino-3-[(5-tritylamino)-pentyl]pyrazole-4-carboxamide.

12 **EXAMPLE 12:**

13 **4-Hydroxy-6-methylthio-3-[(5-tritylamino)pentyl]pyra-**
14 **zolo-[3,4-d]pyrimidine.**

15 The carboxamide from Example 11 is reacted with potassium
16 ethyl xanthate and ethanol at an elevated temperature to give the
17 potassium salt of 4-hydroxypyrazolo[3,4-d]pyrimidine-6-thiol. This salt is
18 then reacted with iodomethane to give 4-hydroxy-6-methylthio-3-
19 [(5-tritylamino)pentyl]pyrazolo[3,4-d]pyrimidine.

20 **EXAMPLE 13:**

21 **1-(2-Deoxy-β-D-erythropentofuranosyl)-4-hydroxy-3-[5-**
22 **(tritylamino)pentyl]pyrazolo[3,4-d]pyrimidin-6-amine.**

23 Following the procedure of Example 5, the pyrazolopyrimidine of
24 Example 12 is treated with sodium hydride and reacted with
25 1-chloro-1,2-dideoxy-3,5-di-O-toluoylribofuranose. The resulting
26 compound is reacted with MCPBA and with methanolic ammonia, and
27 the toluoyl protecting groups are removed to give the product.

28 **EXAMPLE 14:**

29 **1-(2-Deoxy-β-D- erythropent furanosyl)-4-hydroxy-3-[5-**
30 **(6-biotinamido)hexanamidopentyl]pyrazolo[3,4-d]pyrimidin- 6-amine**
31 **5'-monophosphat .**

1 Following the procedure of Example 7, the pyrazolopyrimidine of
2 Example 13 is reacted with phosphoryl chloride to give the
3 corresponding 5'-monophosphate.

4 Following the procedure of Example 8, the above
5 5'-monophosphate is reacted with palladium/carbon and cyclohexadiene,
6 and the residue is reacted with N-hydroxy-succinimidyl
7 biotinylaminocaproate to give
8 1-(2-deoxy-β-D-erythropentofuranosyl)-4-hydroxy-3-[5-
9 (6-biotinamido)hexanamidopentyl]pyrazolo[3,4-d]pyrimidin-6-amine
10 5'-monophosphate.

11 EXAMPLE 15:

12 1-(2-Deoxy-β-D-erythropentofuranosyl)-4-hydroxy-3-[5-
13 (6-biotinamido)hexanamidopentyl]pyrazolo[3,4-d]pyrimidin-6-amine
14 5'-triphosphate.

15 Following the procedure of Example 9, the 5'-monophosphate of
16 Example 14 is treated with carbonyldiimidazole and then reacted with
17 tributylammonium pyrophosphate to give the corresponding
18 5'-triphosphate.

19 EXAMPLE 16:

20 1-(2-Deoxy-β-D-erythropentofuranosyl)-3-[5-(trityl-
21 amino)-pentyl]pyrazolo[3,4-d]pyrimidine-4-benzoylamine.

22 1-(2-Deoxy-β-D-erythropentofuranosyl)-3-[5-(tri-
23 tylamino)pentyl]pyrazolo[3,4-d]pyrimidine-4-amine from Example 6 is
24 reacted with benzoyl chloride and pyridine to give
25 1-(2-deoxy-3,5-di-O-benzoyl-β-D-erythropentofuranosyl)-3-[5-(tritylamino
26)pentyl]pyrazolo- [3,4-d]pyrimidine-4-dibenzoylamine. This is treated
27 with aqueous sodium hydroxide to partially deprotect the compound
28 giving 1-(2-deoxy-β-D-erythropentofuranosyl)-3-[5-(tritylamino)-
29 pentyl]pyra- zolo[3,4-d]pyrimidine-4-benzoylamine.

30 EXAMPLE 17:

31 1-(2-Deoxy-β-D-erythropentofuranosyl)-3-[5-(trifluoro-

1 acetamido)pentyl]pyrazolo[3,4-d]pyrimidine-4-benzoyl- amine.

2 Following the procedure of Example 8, the benzoylamine of
3 Example 16 is treated with palladium hydroxide on carbon and then
4 with trifluoroacetic anhydride to give 1-(2-deoxy-β-D-erythropentofuran-
5 osyl)-3-[5-(trifluoroacetamido)pentyl]pyrazolo[3,4-d]pyrimidine-4-benzoyl
6 amine.

7 EXAMPLE 18:

8 1-(2-Deoxy-5-O-dimethoxytrityl-β-D-erythropentofuran-
9 osyl)-3-[5-(trifluoroacetamido)pentyl]pyrazolo[3,4-d]pyrimidine-4-benzoyl
10 amine 3'-O-(N,N-diisopropyl)phosphoramidite cyanoethyl ester.

11 The compound of Example 17 is reacted with dimethoxytrityl
12 chloride and pyridine to give the corresponding 5'-dimethoxytrityl
13 compound. This compound is then reacted with cyanoethyl
14 chloro-N,N-diisopropylphosphoramidite (according to the method of
15 Sinha et al., Nucleic Acids Res., 12:4539 (1984)) to give the
16 3'-O-activated nucleoside.

17 Synthesis of nucleotides and ODNs including a cross-linking function

18 EXAMPLE 19

19 5-(4-Phthalimidobut-1-yn-1-yl)-2'-deoxyuridine

20 5-Iodo-2'-deoxyuridine (354 mg, 1 mmol) was dissolved in 10 mL
21 of dimethylformamide. Cuprous iodide (76 mg, 0.4 mmol),
22 tetrakis(triphenylphosphine)palladium(0) (230 mg, 0.2 mmol), and
23 triethylamine (200 mg, 2.0 mmol) were added. 4-Phthalimidobut-1-yne
24 (300 mg, 1.5 mmol) was added all at once and the reaction kept at 60°C
25 for three hours. The clear yellow reaction was then evaporated and
26 methylene chloride was added. Scratching of the flask induced
27 crystallization of nearly all of the product which was filtered and
28 recrystallized from 95% ethanol to give 335 mg (78%) of title
29 compound as fine, feathery needles.

30 EXAMPLE 20

31 5-(4-Phthalimidobut-1-yl)-2'-deoxyuridine

1 1.00 Gram of 5-(4-Phthalimidobut-1-yn-1-yl)-2'-deoxyuridine was
2 dissolved in 95% EtOH and about 3 g of neutral Raney nickel was
3 added. After 48 hours, the catalyst was removed by cautious filtration
4 and the filtrate was evaporated to a solid which was recrystallized from
5 methanol-water to give 960 mg (97%) of the title compound.

6 EXAMPLE 21:

7 5-(3-Iodoacetamidopropyl)-2'-deoxyuridine.

8 5-(3-Trifluoroacetamidoprop-1-yl)-2'-deoxyuridine (0.3 mmol) is
9 treated with ammonia and then with N-hydroxy-succinimidyl
10 α -iodoacetate (0.5 mmol). The reaction mixture is evaporated to
11 dryness and purified by chromatography to give
12 5-(3-iodoacetamidopropyl)-2'-deoxyuridine.

13 EXAMPLE 22 5-(4-(4-Bromobutyramido)butyl)-2'-deoxyuridine

14 5-(4-phthalimidobut-1-yl)-2'-deoxyuridine is treated with ammonia
15 and then with N-hydroxysuccinimidyl-4-bromobutyrate to give
16 5-(4-(4-bromobutyramido)butyl)-2'-deoxyuridine.

17 Preparation of Synthetic Oligonucleotides

18 EXAMPLE 23:

19 Phosphoramidite Preparation and DNA Synthesis.

20 Nucleosides were 5'-dimethoxytritylated, following known
21 procedures, to give around 85% yield, and the 3'-phosphoramidite was
22 made using diisopropylamino β -cyanoethylchlorophosphite (as described
23 in "Oligonucleotide Synthesis: A Practical Approach", supra) with
24 diisopropylethylamine in methylene chloride. The phosphoramidite was
25 made into a 0.2N solution in acetonitrile and placed on the automated
26 DNA synthesizer. Incorporation of these new and modified
27 phosphoramidites gave incorporation similar to ordinary
28 phosphoramidites (97-99% as judged by assay of the trityl color released
29 by UV.)

30 Oligonucleotides were removed from the DNA synthesizer in
31 tritylated form and deblocked using 30% ammonia at 55°C for 6 hours.

1 Ten μL of 0.5M sodium bicarbonate was added to prevent acidification
2 during concentration. The oligonucleotide was evaporated to dryness
3 under vacuum and redissolved in 1.0 mL water. The oligonucleotides
4 were purified by HPLC using 15-55% acetonitrile in 0.1N
5 triethylammonium acetate over 20 minutes. Unsubstituted
6 oligonucleotides came off at 10 minutes; amino derivatives took 11-12
7 minutes. The desired oligonucleotide was collected and evaporated to
8 dryness, then it was redissolved in 80% aqueous acetic acid for 90
9 minutes to remove the trityl group. Desalting was accomplished with a
10 G25 Sephadex column and appropriate fractions were taken. The
11 fractions were concentrated, brought to a specific volume, dilution
12 reading taken to ascertain overall yield and an analytical HPLC done to
13 assure purity. Oligonucleotides were frozen at -20°C until use.

14 In general, to add the crosslinking arm to an
15 aminoalkyloligonucleotide, a solution of 10 μg of the
16 aminoalkyloligonucleotide and a 100X molar excess of
17 n-hydroxysuccinimide haloacylate such as α -haloacetate or
18 4-halobutyrate in 10 μL of 0.1 M borate buffer, pH 8.5, is incubated at
19 ambient temperature for 30 min. in the dark. The entire reaction is
20 passed over a NAP-10 column equilibrated with and eluted with distilled
21 water. Appropriate fractions based on UV absorbance are combined
22 and the concentration is determined spectrophotometrically.

23 2,3,5,6-Tetrafluorophenyl trifluoroacetate.

24 A mixture of 2,3,5,6-tetrafluorophenol (55.2 g, 0.33 mol),
25 trifluoroacetic anhydride (60 mL, 0.42 mol) and boron trifluoride
26 etherate (0.5 mL) was refluxed for 16 hr. Trifluoroacetic anhydride and
27 trifluoroacetic acid were removed by distillation at atmospheric
28 pressure. The trifluoroacetic anhydride fraction (bp 40°C) was returned
29 to the reaction mixture along with 0.5 mL of boron trifluoride etherate,
30 and the mixture was refluxed for 24 hr. This process was repeated two
31 times to ensure complete reaction. After distillation at atmospheric

1 pressure, the desired product was collected at 62°C/45 mm (45°C/18
2 mm) as a colorless liquid: yield = 81.3 g (93%); $d = 1.52$ g/mL; $n_D^{21} =$
3 1.3747; IR (CHCl_3) 3010, 1815, 1525, 1485, 1235, 1180, 1110, and 955
4 cm^{-1} . Anal. Calcd for $\text{C}_8\text{HF}_7\text{O}_2$: C, 36.66; H, 0.38; F, 50.74. Found: C,
5 36.31; H, 0.43; F, 50.95.

6 2,3,5,6-Tetrafluorophenyl-4'-[bis(2-chloroethyl)amino]phenylbutyrate
7 (Chlorambucil 2,3,5,6-tetrafluorophenyl ester)

8 To a solution of 0.25 g (0.82 mmol) of chlorambucil (supplied by
9 Fluka A. G.) and 0.3 g (1.1 mmol) of 2,3,5,6-tetrafluorophenyl
10 trifluoroacetate in 5 ml of dry dichloromethane was added 0.2 ml of dry
11 triethylamine. The mixture was stirred under argon at room temperature
12 for 0.5 h and evaporated. The residual oil was purified by column
13 chromatography on silica gel with hexane-chloroform (2:1) as the eluting
14 solvent to give the ester as an oil: 0.28 g (75%); TLC on silica gel
15 (CHCl_3) R_f 0.6; IR (in CHCl_3) 3010, 1780, 1613, 1521, 1485 cm^{-1} .
16 2-Propargyloxyethyl)amine (John, R., and Seitz, G., Chem. Ber., 123,
17 133 (1990) was prepared by condensing propynol with
18 2-bromoethylammonium bromide in liquid ammonia in the presence of
19 Na NH_2 , and was used crude for the next reaction.

20 3-(2-Trifluoroacetamidoethoxy)propyne

21 (2-Propargyloxyethyl)amine (13.8 g, 0.14 mol) is stirred and
22 chilled in an iso-propanol-dry ice bath while excess of trifluoroacetic
23 anhydride (26 ml, 0.18 mol) is added dropwise.
24 N-(2-Propargyloxyethyl)trifluoroacetamide is distilled at 84-85°/1.7 torr
25 as an oil which solidified upon refrigeration; yield 14.4 g (52%), m.p.
26 (16°, n_D^{24} 1.4110. Anal. Calcd. for $\text{C}_7\text{H}_8\text{F}_3\text{NO}_2$: C, 43.09, H, 4.13; N,
27 7.18; F, 29.21. Found: C, 42.80; H, 4.03; N, 7.06; F, 29.38.

28 5-[3-(2-Trifluoroacetamidoethoxy)propynyl]-2'-deoxyuridine A

29 mixture of 5-iodo-2'-deoxyuridine (3.54 g, 10 mmol), copper(I) iodide
30 (0.19 g, 1 mmol) and tetrakis(triphenylphosphine)palladium(0) (0.58 g,
31 0.5 mmol) is dried in vacuo at 60° for 3 hours and placed under argon.

1 A suspension of the mixture in dry DMF (20 ml) is stirred under argon
2 and treated with dry triethylamine (1.7 ml, 12 mmol) followed by
3 3-(2-Trifluoroacetamidoethoxy)propyne (3.17 g, 16 mmol). The mixture
4 is cooled at room temperature in a water bath and stirred for 17 hours.
5 The mixture is treated with 2% acetic acid (100 ml), the catalyst is
6 removed by filtration and washed with 50% methanol. The filtrates are
7 combined and passed onto a LiChroprep RP-18 column (5X25 cm), the
8 column is washed, then eluted with 1% acetic acid in 50% (v/v)
9 methanol. The fractions with the main product are combined,
10 evaporated, and dried in vacuo. The resultant foam is stirred with 150
11 ml of ether to give crystalline product; yield 3.6 g (85%); m.p. 145-152°.
12 5-[3-(2-Trifluoroacetamidoethoxy)propyl]2'-deoxyuridine

13 A solution of 5-[3-(2-trifluoroacetamidoethoxy)-
14 propynyl]-2'-deoxyuridine (3.4 g, 8.1 mmol) in methanol (20 ml) is
15 stirred with ammonium formate (prepared by addition of 3 ml, 79 mmol
16 of cold 98% formic acid into 2 ml, 50 mmol of dry ice frozen 25%
17 ammonia) and 0.2 g of 10% Pd/C for 7 hours at room temperature
18 under hydrogen atmosphere. The catalyst is removed by filtration, the
19 filtrate evaporated and product is purified on LiChroprep RP-18 column
20 by the above procedure. Fractions containing the desired product are
21 combined and evaporated to dryness in vacuo and the resultant solid is
22 triturated with dry ether to give 3.0 g (87% product, m.p. 107-110°; _{max}
23 in nm, in 0.1M triethylamine-acetate (pH 7.5), 220, 268. Analysis
24 calculated for C₁₆H₂₂F₃N₃O₇: C, 45.18; H, 5.21; N, 9.88; F, 13.40. Found
25 C, 45.16; H, 5.16; N, 9.68; F, 13.13.

26 Introduction of chlorambucil residue into the primary amino groups of
27 oligonucleotides

28 Preparation of the cetyltrimethylammonium salt of oligonucleotides: a
29 100 µL aliquot of aqueous solution of oligonucleotide (50-500 ug),
30 generally triethylammonium salt, was injected to a column packed with
31 Dowex 50wx8 in the cetyltrimethylammonium form and prewashed with

1 50% alcohol in water. The column was eluted by 50% aqueous ethanol
2 (0.1 mL/min). Oligonucleotide containing fraction was dried on a
3 Speedvac over 2 hours and used in following reactions.

4 Ethanol solution (50 uL) of cetyltrimethylammonium salt of an
5 oligonucleotide (50-100 µg) was mixed with 0.08 M solution of
6 2,3,5,6-tetrafluorophenyl-4'-[bis(2-chloroethyl)amino]phenylbutyrate
7 (tetrafluorophenyl ester of chlorambucil) in acetonitrile (50 µL) and 3
8 µL of diisopropylethylamine. After shaking for three hours at room
9 temperature, the product was precipitated by 2% LiClO₄ in acetone (1.5
10 mL). The product was reprecipitated from water (60 uL) by 2% LiClO₄
11 in acetone three times. Finally the chlorambucil derivative of the
12 oligonucleotide was purified by Reverse Phase Chromatography with
13 approximately 50-80% yield. The fraction containing the product was
14 concentrated by addition of butanol. The isolated chlorambucil
15 derivative of the oligonucleotide was precipitated in acetone solution
16 with LiClO₄, washed by acetone and dried under vacuum. All
17 manipulations of reactive oligonucleotide were performed as quickly as
18 possible, with the product in ice-cold solution.

19 Preparation of SBC ODNs

20 N-phenoxyacetyl protected 2'-deoxyguanosine and 2'-deoxycytidine
21 3'-O-2-cyanoethyl-N,N'-diisopropylphosphoramidites are available
22 commercially from BioGenex, Alameda, California.
23 5'-O-dimethoxytrityl-2-thiothymi-
24 dine-3'-O-(2-cyanoethyl-N,N'-diisopropylphosphoramidite) was prepared
25 using the procedure of Connolly et al. supra.
26 2,6-diaminopurine-2'-deoxyribose was synthesized as described by
27 Fathi et al. supra.
28 N²,N⁶-bis(phenoxyacetyl)-2,6-diaminopurine-2'-deoxyribose
29 (Comp und 1, Reaction Scheme 1).

30 This compound is prepared substantially in accordance with the
31 literature procedure of Schulhof et al.(1987) Nucleic Acids Res. 15,

1 397-416. 2,6-Diaminopurine-2'-deoxyriboside (1.8 g, 6.8 mmol) is dried
2 by evaporation with dry pyridine. Trimethylchlorosilane (5 mL, 39
3 mmol) is added dropwise to an ice cold solution of
4 2,6-diaminopurine-2'-deoxyriboside in 35 mL of dry pyridine. After 30
5 min, phenoxyacetic anhydride (8.0 g, 28 mmol) is added to the stirred
6 solution. The mixture is kept for 3 h at RT then cooled to 5°C. Water
7 (5 mL) is added to quench the excess of phenoxyacetic anhydride. After
8 being stirred for 2 h, the reaction mixture is concentrated on a rotary
9 evaporator to approximately 10 mL and then diluted with water to 120
10 mL to give an emulsion. The emulsion is washed with ether (150 mL).
11 The resulting precipitate is filtered, washed with ether, water, and dried
12 in vacuo. The material (3.2 g, 87 %) obtained by using this procedure is
13 pure enough to be used in the next step without additional purification.
14 5'-O-Dimethoxytrityl-N²,N⁶-bis(phenoxyacetyl)-2,6-diaminopurine-2'-deox
15 yriboside (Compound 2).

16 N²,N⁶-bis(phenoxyacetyl)-2,6-diaminopurine-2'-deoxyriboside (3.2
17 g, 5.8 mmol) is dried by evaporation with dry pyridine (2x20 mL) and
18 dissolved in 30 mL of the same solvent. 4,4'-dimethoxytrityl chloride
19 (2.0 g, 6 mmol) is added in one portion with vigorous stirring. After 1 h,
20 TLC (CHCl₃/MeOH, 19:1 v/v) indicates complete reaction. The
21 reaction mixture is concentrated on a rotary evaporator and diluted with
22 dichloromethane to approximately 200 mL. After being washed with
23 saturated NaHCO₃ (2x200 mL), the organic layer is dried with Na₂SO₄
24 and then concentrated in vacuo to an oil. Preparative silica gel
25 chromatography with a gradient of MeOH in CH₂Cl₂ from 0 to 5%
26 provides the desired product as a crystalline solid (3.3 g, 68%).
27 5'-O-Dimethoxytrityl-N²,N⁶-bis(phenoxyacetyl)-2,6-diaminopurine-2'-deox
28 yriboside-3'-O-(2-cyanoethyl-N,N'-diisopropylphosphoramidite)
29 (Compound 3).

30 A suspension of Compound 2 (3.1 g, 3.7 mmol) in a mixture of

1 dichloromethane (30 mL) and diisopropylethylamine (4 mL) is treated
2 with 2-cyanoethoxy N,N-diisopropylaminochlorophosphine (1.6 mL, 7.2
3 mmol). The reaction is stirred for 1 h, and quenched by addition of
4 methanol (0.1 mL). After 2 min, dichloromethane (70 mL) is added and
5 the solution is washed with 1 M NaHCO₃ (100 mL) followed by
6 saturated brine (100 mL). The organic layer is dried, filtered, and the
7 solvent is removed in vacuo. The crude product is purified by
8 preparative silica gel chromatography (ethyl
9 acetate/dichloromethane/triethylamine, 45:45:5 v/v/v). After the
10 purification the product is additionally precipitated in hexane to give a
11 colorless solid (2.5 g, 65 %).

12 N,N,N'-tris(9-fluorenylmethoxycarbonyl)-2,6-diaminopu-
13 rine-2'-deoxyriboside (Compound 4, Reaction Scheme 2)

14 2,6-Diaminopurine-2'-deoxyriboside (2.3 g, 8.5 mmol) is dried by
15 evaporation with dry pyridine and dissolved in 40 mL of the same
16 solvent. Trimethylchlorosilane (5 mL, 3.9 mmol) is added dropwise to
17 the ice cold solution and the reaction is kept for 1 min at 5°C and 15
18 min at RT. 9-Fluorenylmethoxy carbonyl chloride (6.2 g, 24 mmol) is
19 added, and the reaction mixture is stirred for 2 h. Hydrolysis of the
20 trimethylsilyl groups and of excess chlorides is effected by addition of
21 water (30 mL). After stirring for 18 h, the mixture is evaporated to near
22 dryness and co-evaporated with toluene to remove residual pyridine.
23 Upon addition of water (150 mL) a white solid is precipitated. The
24 suspension is shaken with ether (100 mL) and is then filtered to give an
25 off-white solid. TLC (CHCl₃/MeOH, 9:1 v/v) shows at least three new
26 products. The major product with higher R_f is isolated by silica gel
27 chromatography using a gradient of methanol in dichloromethane. The
28 product is a white solid (1.2 g, 15%).

29 5'-O-Dimethoxytrityl-N,N,N'-tris(9-fluorenylmethoxycarbonyl)-2,6-diamin
30 opurine-2'-deoxyriboside (Compound 5).

31 The title compound is prepared in accordance with the procedure

1 described for the phenoxyacetylated analog in 70% yield.
2 5'-O-Dimethoxytrityl-N,N,N'-tris(9-fluorenylmethoxycarbonyl)-2,6-diamin
3 opurine-2'-deoxyribose-3'-O-(2-cyanoethyl-N,N'-diisopropylphosphora
4 midite) (Compound 6).

5 The general method demonstrated described for the
6 phenoxyacetylated analog Compound 3 (see above) is used to
7 synthesize this phosphoramidite.

8 Preparation of hexanol-oxalyl Primer Support

9 This support is made by analogy to the literature method (Alul et
10 al. Nucleic Acids Res. (1991) 19, 1527-1532). Solution I is prepared by
11 dissolving of 2.8 g (6.7 mmol) of O-(4,4'-dimethoxytrityl)-1,6-hexanediol
12 (5) in dry acetonitrile (8 mL). To prepare solution II, oxalyl chloride
13 (0.6 mL) is added to a stirred solution of 1,2,4-triazole (2.1 g, 30 mmol)
14 in 60 mL of acetonitrile, then pyridine (2 mL) is added to dissolve the
15 resulting precipitate. Solution I is added dropwise to Solution II with
16 stirring. After 1 h, amino modified Primer Support (20 g) (Pharmacia) is
17 added in one portion. The suspension is swirled on a rotary shaker for
18 15 min, then filtered on a sintered glass filter, washed with methanol
19 (200 mL), acetone (500 mL) and ether (200 mL). After being dried for
20 30 min in vacuo, the support is treated with a mixture of pyridine (60
21 mL), acetic anhydride (6 mL), and N-methyl imidazole (6 mL). After
22 15 min, the support is filtered, washed as described above, and dried in
23 vacuo overnight. The product is analyzed for dimethoxytrityl content
24 according to the literature method (Atkinson, T., and Smith, M., in
25 "Oligonucleotide Synthesis, A Practical Approach", M. Gait, Ed., IRL
26 Press, Washington, D. C. pp 35-81 (1984)), and in the specific example
27 was found to have a loading of 32 $\mu\text{mol/g}$.

28 Oligonucleotide synthesis. Oligonucleotide synthesis is performed
29 on a Pharmacia OligoPilot DNA synthesiz r in 10 μmol scale using
30 either hexanol Primer Support which was prepared accordingly to the

1 procedure described for hexanol CPG (Gamper et al.(1993) Nucleic
2 Acids Res. 21, 145-150) or hexanoloxyalyl Primer Support described
3 above.

4 For the preparation of oligonucleotides containing
5 2-thiothymidine and 2-aminoadenosine, two alternative methods can be
6 used. In the first method, N-phenoxyacetyl protected
7 5'-O-dimethoxytrityl-2'-deoxynucleoside-2-cyanoethyl-N,N'-diisopropylami
8 ne-phosphoramidites are used. DNA synthesis cycle is carried out as for
9 regular phosphoramidites. Time of the deprotection with concentrated
10 ammonia is reduced to 2 h at 50°C. In the second method, Fmoc
11 protected phosphoramidites are employed. The synthesis is performed
12 using the hexanol-oxyalyl Primer Support and the standard DNA
13 synthesis cycle, with the exception of the capping step which is omitted.
14 Deprotection is carried out by treatment of the solid support with 0.2 M
15 1,8-diazobicyclo[5.4.0]undec-7-ene (DBU) in DMF for 5 min followed
16 by 10% ammonia for additional 5 min.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Microprobe Corporation
Bothell, WA 98021
- (ii) TITLE OF INVENTION: SELECTIVE BINDING COMPLEMENTARY
OLIGONUCLEOTIDES
- (iii) NUMBER OF SEQUENCES: 8
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Klein & Szekeres
 - (B) STREET: 4199 Campus Drive, Suite 700
 - (C) CITY: Irvine
 - (D) STATE: CA
 - (E) COUNTRY: USA
 - (F) ZIP: 92715
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/539,097
 - (B) FILING DATE: 04-OCT-1995
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Szekeres, Gabor L.
 - (B) REGISTRATION NUMBER: 28,675
 - (C) REFERENCE/DOCKET NUMBER: 491-10-PA
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 714-854-5502
 - (B) TELEFAX: 714-854-4897

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ix) FEATURE:
 - (A) NAME/KEY: misc_feature
 - (B) LOCATION: 1..28
 - (D) OTHER INFORMATION: /note= "corresponds to "Watson"
strand of Hybrids I & III"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGACAACGA TCGGAGGACC GAAGGAGC

28

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..28
- (D) OTHER INFORMATION: /note= "corresponds to "Crick"
strand of Hybrids I & II"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCTCCTTCGG TCCTCCGATC GTTGTCAG

28

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: one-of(1, 5, 8, 12, 19, 20, 28)
- (D) OTHER INFORMATION: /mod_base= OTHER
/note= "pyrrolo-[2,3-d]pyrimidine-2(3H)-one"

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: one-of(3, 9, 13, 14, 16, 17, 21, 24, 25, 27)
- (D) OTHER INFORMATION: /mod_base= OTHER
/note= "hypoxanthine"

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..28
- (D) OTHER INFORMATION: /note= "corresponds to "Watson"
strand of Hybrids II & IV"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

NTNANAANNA TNNNANNANN NAANNANN

28

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: one-of(1, 9, 10, 17, 21, 24, 28)
- (D) OTHER INFORMATION: /mod_base= OTHER
/note= "hypoxanthine"

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: one-of(2, 4, 5, 8, 12, 13, 15, 16, 20, 26)
- (D) OTHER INFORMATION: /mod_base= OTHER
/note= "pyrrolo-[2,3-d]pyrimidine-2(3H)-one"

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..28
- (D) OTHER INFORMATION: /note= "corresponds to "Crick"
strand of Hybrids III & IV"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

NNTNNTTNNN TNNTNNNATN NTTNTNAN

28

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTAAGAGAAT TATGCAGTGC

20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCACTGCATA ATTCTCTTAC

20

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: one-of(2, 10, 11, 13, 18)
- (D) OTHER INFORMATION: /mod_base= OTHER
/note= "d2sThymine replaces all dThymine"

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: one-of(3, 4, 6, 8, 9, 12, 16)
- (D) OTHER INFORMATION: /mod_base= OTHER
/note= "d2amAdenine replaces all dAdenine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTAAGAGAAT TATGCAGTGC

20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: one-of(3, 8, 10, 11, 19)
- (D) OTHER INFORMATION: /mod_base= OTHER
/note= "d2amAdenine replaces all dAdenine"

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: one-of(5, 9, 12, 13, 15, 17, 18)
- (D) OTHER INFORMATION: /mod_base= OTHER
/note= "d2sThymine replaces all dThymine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCACTGCATA ATTCTCTTAC

20

WHAT IS CLAIMED IS:

1
2 **1.** A pair of oligonucleotides (ODNs), each of said ODNs
3 comprising nucleotide moieties having naturally occurring aglycon bases
4 and a combination of modified aglycon bases selected from the group
5 consisting of the combinations (1) A', T', (2) G', C' and (3) A', T', G',
6 C', the duplex form of said pair of ODNs having a melting temperature
7 under physiological conditions of less than approximately 40°C, each of
8 said pair of ODNs being substantially complementary in the
9 Watson-Crick sense to one of the two strands of a duplexed target
10 sequence in nucleic acid,

11 wherein the nucleotide moieties having the modified bases have
12 the following properties:

13 within complementary oligonucleotides A' does not form a
14 stable hydrogen bonded base pair with T' and forms a stable hydrogen
15 bonded base pair with T;

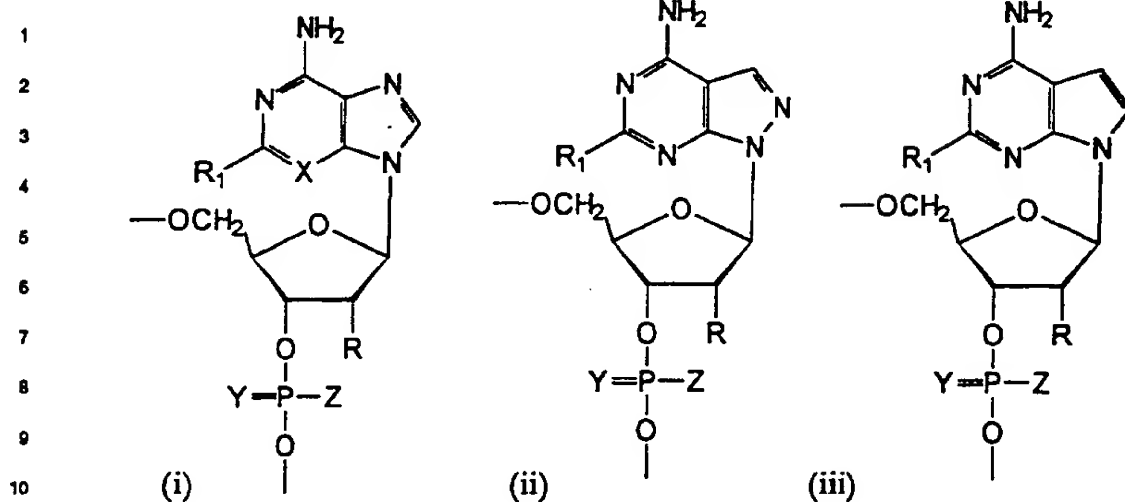
16 within complementary oligonucleotides T' does not form a stable
17 hydrogen bonded base pair with A' and forms a stable hydrogen bonded
18 base pair with A;

19 within complementary oligonucleotides G' does not form a stable
20 hydrogen bonded base pair with C' and forms a stable hydrogen bonded
21 base pair with C, and

22 within complementary oligonucleotides C' does not form a stable
23 hydrogen bonded base pair with G' and forms a stable hydrogen bonded
24 base pair with G, wherein the pair of oligonucleotides are optionally
25 linked to one another by a covalently bonded tether.

26 **2.** The ODNs of Claim 1 wherein the nucleotide moiety A'
27 has the structure selected from the groups shown by formulas (i), (ii)
28 and (iii)

59



wherein

X is N or CH;

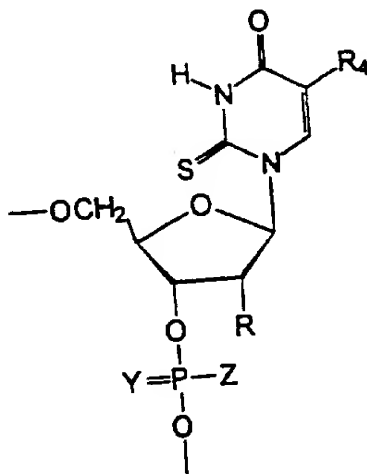
Y is O or S;

Z is OH or CH₃;

R is H, F, or OR₂, where R₂ is H, C₁₋₆ alkyl or allyl, and

R₁ is C₁₋₄ alkyl, C₁₋₄ alkoxy, C₁₋₄ alkylthio, F, or NHR₃, where R₃ is H, or C₁₋₄ alkyl, and where the 8 position of the purine, the 3 position of the pyrazolopyrimidine or the 5 position of the pyrrolopyrimidine optionally serve as a point of attachment for a cross-linking function or a reporter group.

3. The ODNs of Claim 1 wherein the nucleotide moiety T' has the formula (iv)



(iv)

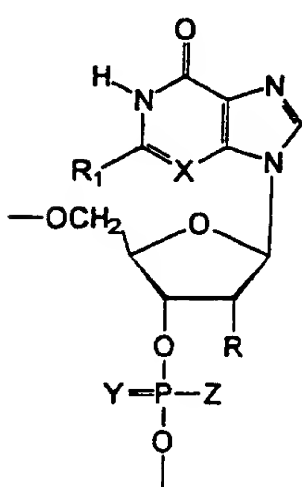
wherein

Y is O or S;

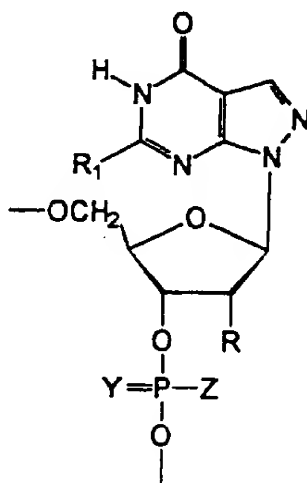
Z is OH or CH₃;R is H, F, or OR₂, where R₂ is H, C₁₋₆ alkyl or allyl, and

R₄ is H, C₁₋₆ alkyl, C₁₋₆ alkenyl, C₁₋₆ alkynyl, or optionally the 5-position of the pyrimidine serves as a point of attachment for a cross-linking function or a reporter group.

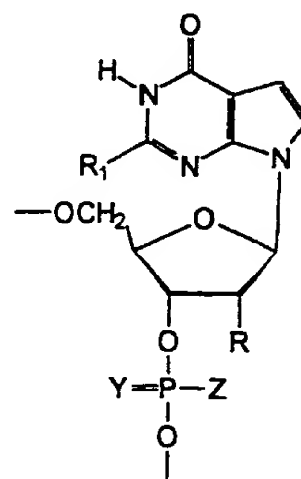
4. The ODNs of Claim 1 wherein the nucleotide moiety G' has the structure selected from the groups shown by formulas (v), (vi) and (vii)



(v)



(vi)



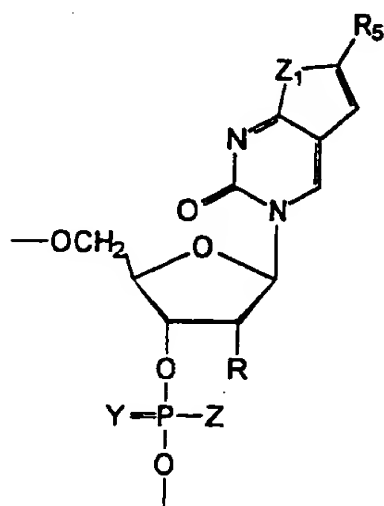
(vii)

wherein

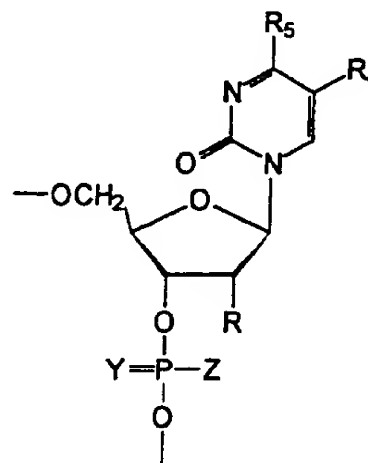
X is N or CH;

- 1 Y is O or S;
 2 Z is OH or CH₃;
 3 R is H, F, or OR₂, where R₂ is H, C₁₋₆ alkyl or allyl, and
 4 R₁ is H, C₁₋₄ alkyl, C₁₋₄ alkoxy, C₁₋₄ alkylthio, F, or NHR₃, where
 5 R₃ is H, or C₁₋₄ alkyl, and where the 8 position of the purine, the 3
 6 position of the pyrrazolopyrimidine or the 5 position of the
 7 pyrrolopyrimidine optionally serve as a point of attachment for a
 8 cross-linking function or a reporter group.

9 5. The ODNs of Claim 1 wherein the nucleotide C' has the
 10 structure selected from the groups shown by formulas (viii) and (ix)



(viii)



(ix)

24 wherein

- 25 Y is O or S;
 26 Z is OH or CH₃;
 27 R is H, F, or OR₂, where R₂ is H, C₁₋₆ alkyl or allyl,
 28 R₄ is H, C₁₋₆ alkyl, C₁₋₆ alkenyl, C₁₋₆ alkynyl, or optionally the
 29 5-position of the pyrimidine serves as a point of attachment for a
 30 cross-linking function or a reporter group;

- 1 Z₁ is O or NH, and
2 R₅ is H, or C₁ - 4 alkyl.
- 3 6. The ODNs of Claim 2 wherein the nucleotide moiety A'
4 has the structure in accordance with formula (I).
- 5 7. The ODNs of Claim 6 wherein X is N, Z is OH, and Y is
6 O.
- 7 8. The ODNs of Claim 7 wherein R₁ is NH₂.
- 8 9. The ODNs of Claim 3 wherein Z is OH, and Y is O.
- 9 10. The ODNs of Claim 9 wherein R₄ is CH₃.
- 10 11. The ODNs of Claim 4 wherein the nucleotide moiety G'
11 has the structure in accordance with formula (v).
- 12 12. The ODNs of Claim 11 wherein X is N, Z is OH, and Y is
13 O.
- 14 13. The ODNs of Claim 12 wherein R₁ is H.
- 15 14. The ODNs of Claim 5 wherein the nucleotide moiety C'
16 has the structure in accordance with formula (viii).
- 17 15. The ODNs of Claim 14 wherein , Z is OH, Z₁ is NH and Y
18 is O.
- 19 16. The ODNs of Claim 15 wherein R₅ is H.
- 20 17. The ODNs of Claim 1 having approximately 5 to 99
21 nucleotide units.
- 22 18. The ODNs of Claim 1 wherein each of the nucleotides is a
23 2'-deoxyribonucleotide.
- 24 19. The ODNs of Claim 1 wherein each of the nucleotides is a
25 ribonucleotide.
- 26 20. The ODNs of Claim 1 comprising at least one nucleotide
27 unit having a 2-O-methylribose moiety.
- 28 21. The ODNs of Claim 1 comprising a cross-linking agent
29 covalently attached to at least one nucleotide unit.
- 30 22. The ODNs of Claim 1 comprising a reporter group.

1 **23.** The ODNs of Claim 1 wherein the combination of
2 modified aglycon bases is A', T'.

3 **24.** The ODNs of Claim 1 wherein the combination of
4 modified aglycon bases is G', C'.

5 **25.** The ODNs of Claim 1 wherein the combination of
6 modified aglycon bases is A', T', G', C'.

INTERNATIONAL SEARCH REPORT

International Application No.

PC 1/US 96/15934

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H21/00 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07H C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STUDIA BIOPHYSICA, vol. 55, no. 1, 1976, pages 21-27, XP002024408 K. H. SCHEIT ET AL: "Stereochemical Basis of Template Function" see page 23 - page 24 see page 22; figure 1 --- -/-	1-3, 6-10, 17-23,25

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

Z document member of the same patent family

Date of the actual completion of the international search

3 February 1997

Date of mailing of the international search report

10.02.1997

Name and mailing address of the ISA

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Watchorn, P

INTERNATIONAL SEARCH REPORT

International Application No

PC./US 96/15934

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 108, no. 21, 23 May 1988 Columbus, Ohio, US; abstract no. 187183, H. INOUE ET AL: "Synthesis of Dodecadeoxyribonucleotides Containing a Pyrrolo[2,3-d]pyrimidine Nucleoside and their Base-Pairing Ability" page 752; column 1; XP002024262 see abstract & NIPPON KAGAKU KAISHI, no. 7, 1987, pages 1214-1220, ---	1,4,5, 11-22, 24,25
A	DATABASE WPI Section Ch, Week 8750 Derwent Publications Ltd., London, GB; Class B02, AN 87-352165 XP002024264 & JP 62 255 499 A (TEIJIN KK) , 7 November 1987 see abstract & PATENT ABSTRACTS OF JAPAN vol. 012, no. 139 (C-491), 27 April 1988 & JP 62 255499 A (TEIJIN LTD), 7 November 1987, see abstract ---	1,4,5, 11-22, 24,25
A	NUCLEIC ACIDS RESEARCH, vol. 22, no. 2, 25 January 1994, OXFORD GB, pages 131-136, XP002024256 S. CASE-GREEN ET AL: "Studies on the base pairing properties of deoxyinosine by solid phase hybridisation to oligonucleotides" see the whole document ---	1,4,5, 11-22, 24,25
A	NUCLEIC ACIDS RESEARCH, vol. 13, no. 24, 20 December 1985, OXFORD GB, pages 8927-8938, XP002024257 F. H. MARTIN ET AL: "Base pairing involving deoxyinosine: implications for probe design" see the whole document ---	1,4,5, 11-22, 24,25

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INTERNATIONAL SEARCH REPORT

International Application No.

PC, /US 96/15934

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>NUCLEIC ACIDS RESEARCH, vol. 16, no. 1, 11 January 1988, OXFORD GB, pages 305-317, XP002024258 A. CHOLLET ET AL: "DNA containing the base analogue 2-aminoadenine: preparation, use as hybridization probes and cleavage by restriction endonucleases" see the whole document ---</p>	1-3, 6-10, 17-23,25
A	<p>WO 95 14707 A (ISIS PHARMACEUTICALS INC) 1 June 1995 see page 11, paragraph 2 ---</p>	1-3, 6-10, 17-23,25
A	<p>NUCLEIC ACIDS RESEARCH, vol. 22, no. 8, 25 April 1994, OXFORD GB, pages 1429-1436, XP002024259 R. KUIMLEIS ET AL: "Synthesis of oligodeoxynucleotides containing 2-thiopyrimidine residues - a new protection scheme" see page 1430, compounds 1a, 1b; page 1432, table 1 and page 1435, column 1, paragraph 3 ---</p>	1-3, 6-10, 17-23,25
A	<p>CHEMICAL ABSTRACTS, vol. 116, no. 13, 30 March 1992 Columbus, Ohio, US; abstract no. 129471, T. ISHIKAWA ET AL: "Synthesis and Properties of Oligothymidylate Containing Sulfur-Modified Thymidine : Effect of Thiation of Pyrimidine Ring on Thermostability and Conformation of the Duplex" page 949; column 2; XP002024263 see abstract & BIOORG. MED. CHEM. LETT., vol. 1, no. 10, 1991, pages 523-526, ---</p>	1-3, 6-10, 17-23,25

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INTERNATIONAL SEARCH REPORT

International Application No
PC 1/US 96/15934

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>BIOCHEMISTRY, vol. 29, no. 42, 23 October 1990, EASTON, PA US, pages 9891-9901, XP002024260 P. C. NEWMAN ET AL: "Incorporation of a Complete Set of Deoxyadenosine and Thymidine Analogues Suitable for the Study of Protein Nucleic Acid Interactions into Oligonucleotides. Application to the EcoRV Restriction Endonuclease and Modification Methylase" see page 9895; figure 2 see page 9896; tables I,II ---</p>	<p>1-3, 6-10, 17-23,25</p>
A	<p>JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 113, no. 13, 19 June 1991, DC US, pages 5109-5111, XP002024261 P. L. RICHARDSON ET AL: "Tethered Oligonucleotides. A Strategy for the Recognition of Structured RNA" see the whole document ---</p>	<p>1-25</p>
A	<p>WO 95 05391 A (CHROMAGEN INC) 23 February 1995 see figure 14 ---</p>	<p>1-34</p>
P,X	<p>NUCLEIC ACIDS RESEARCH, vol. 24, no. 13, 1 July 1996, pages 2470-2475, XP000621694 WOO J ET AL: "G/C-MODIFIED OLIGODEOXYNUCLEOTIDES WITH SELECTIVE COMPLEMENTARITY: SYNTHESIS AND HYBRIDIZATION PROPERTIES" see the whole document -----</p>	<p>1,4,5, 11-18, 21,24</p>

INTERNATIONAL SEARCH REPORT

I national application No.

PCT/US 96/ 15934

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Claims searched incompletely: 1-22
Please see attached sheet ./,

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/15934

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9514707	01-06-95	US-A- 5459255	17-10-95
		EP-A- 0731807	18-09-96
		US-A- 5587469	24-12-96

WO-A-9505391	23-02-95	CA-A- 2145750	23-02-95
		EP-A- 0669928	06-09-95
